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Highlights

- The microscopic stochastic theory of chromatography is revisited.
- The capability of this theory to handle discrete adsorption frequency distributions is discussed.
- Discrete adsorption frequency distributions are gathered by single-molecule studies.
- Modeling of rare events at molecular viewpoint provides new fundamental information.
- Examples of new insights into solid–liquid interfacial processes are discussed.

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Microscopic models of liquid chromatography: From ensemble-averaged information to resolution of fundamental viewpoint at single-molecule level

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ABSTRACT

In this study, a microscopic probabilistic model of chromatography that establishes a conceptual link between single-molecule dynamics observation at liquid–solid interfaces and chromatographic experiments is described. This model is based on the discrete Lévy representation of stochastic processes and has the great advantage that it can be directly applied to the raw data set of single-molecule observations. The information contained in the molecular measurements includes some erratic rare events that are potentially very informative. Because experimental data need not be processed by mathematical–statistical transformation, application of this model preserves all the information that could be lost in an ensemble-averaged representation. In this approach, single-molecule experiments and stochastic interpretation are combined. It is of great importance to investigate superficial and interfacial phenomena in different areas, such as adsorption mechanisms in chromatography and mechanisms of biological activity, and to track the behavior of individual molecules in living cells.

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1. Introduction

The basis of any microscopic model of chromatography is the description of the behavior of a lone molecule during its migration through a chromatographic column. This is different from the macroscopic approach, which considers the evolution of chromatographic bands in terms of bulk transport properties. The pathway of a single molecule is determined by a number of statistical processes, including the Brownian motion responsible for molecular diffusion, adsorption/desorption process on the stationary phase and flow pattern effects (flow unevenness) in the column [1]. Microscopic (molecular) models of chromatography have also been defined as random or stochastic models. As pointed out by Feller [2], however, the terms random and stochastic are essentially equivalent, the latter is more appropriate when time is a variable, as in chromatography. Because the statistical fluctuations ~~arising from~~ the average ~~exhibited by a molecule~~ are the origin of zone broadening [3], the basis to understand and model

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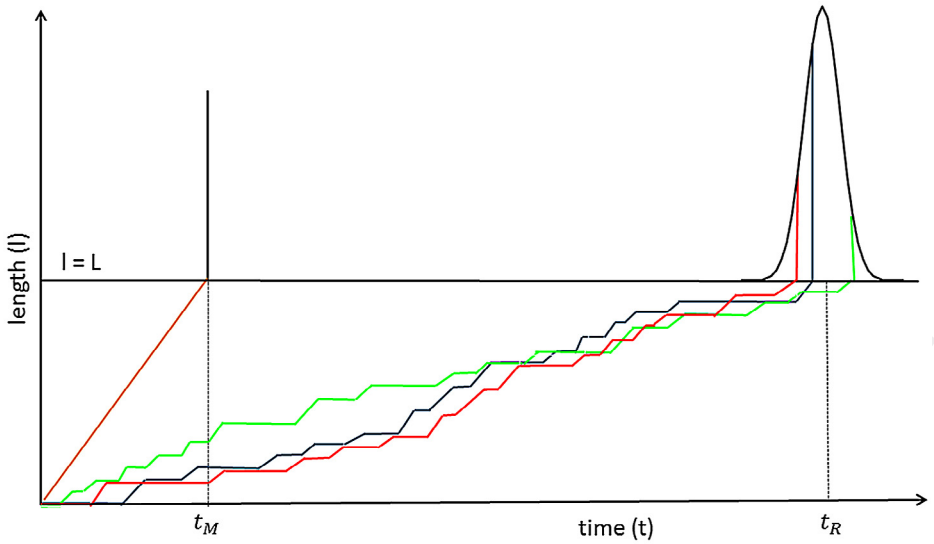

Fig. 1. Graphical representation of chromatography as a stochastic process on a time (t)–length (l) bidimensional plot. Black continuous line: the chromatographic peak as the probability density function of the residence times of molecules in the column. Broken colored lines: examples of random trajectories followed by molecules during their migration through the column. L: column length; t_M: retention time of unretained molecules (if mobile-phase dispersion is absent); t_R: retention time of the chromatographic peak. See text for details.

the dynamic character of chromatography is the study of the chaotic pathway of a single molecule.

Fig. 1 shows a simple chromatographic separation as a stochastic process~~, that is,~~ when molecular diffusion in the mobile phase is neglected. The same process was proposed in the original description of the stochastic theory by Giddings and Eyring in their study in 1955 [4]. In this figure, migration pathways of several molecules are shown as a bidimensional plot in the time–space (t, l) plane during their separation in the chromatographic column. The broken colored lines pass from the moment of injection (at $t = 0$ and $l = 0$) to the elution of molecules from the column (at $t = t_R$ and $l = L$, where L is the length of the column). ~~These~~ trajectories are commonly referred to as molecular histories ~~in stochastic processes~~. Each history is made by the combination of many segments whose slope is either zero (i.e., parallel to the time axis) or equal to the mobile-phase velocity (u_0). These segments represent the two states in which a molecule can be found in the column. The segments parallel to the time axis represent the states of immobilized adsorption on the stationary phase (during which no progression in length occurs), whereas the ones with constant slope (u_0) are the times spent by molecules in the mobile phase, where they identically move at the mobile-phase velocity (in the absence of mobile-phase diffusion). Both the number and lengths of segments are random variables, and hence – from a statistical viewpoint – the history of a molecule is the sum of random numbers (number of adsorption/desorption steps) of random variables (times spent by molecules during the adsorption/desorption events and times spent in the mobile phase between two successive adsorption/desorption steps).

In Fig. 1, the first peak (at t_M) represents the retention time of an unretained compound that travels through the column without performing any adsorption/desorption process. This peak is illustrated as a Dirac delta function, because by neglecting molecular diffusion in the mobile phase, all the unretained molecules move at the same velocity of the mobile phase and are thus eluted exactly at the same time; furthermore, an infinitely narrow injection profile is assumed. On the contrary, the chromatographic peak of a retained compound (peak #2 in Fig. 1) can be interpreted as the probability density function of either trajectory cross sections with the horizontal axis located at $l = L$ or the times spent by molecules in the chromatographic column [5]. When the number of adsorption/desorption steps is large enough (or under the so-called long-time assumption, described later) and the surface is homogeneous [6,7], the shape of this peak tends to a Gaussian distribution, according to the central limit theorem of probability theory [8].

Since the very beginning, one of the most relevant aspects that can be recognized in most of the chromatographic models is the tendency to develop asymptotic theories. This is driven by the concept that an effective chromatographic operation needs sufficient time to produce well-separated peaks. When this is achieved (long-time assumption), an important approximation can be assumed that adsorption/desorption kinetics occur with only a slight deviation from equilibrium [1]. Under the long-time assumption, the convergence of microscopic and macroscopic models has been demonstrated both theoretically [9] and numerically [10].

The stochastic theory of chromatography has been applied to one-, two-, and multisite adsorption processes [7], diffusion-controlled processes [11–13], and different modes of chromatography, including size-exclusion chromatography [14–16], reaction and dynamic chromatography [17–19], ion-exchange chromatography [20], reversed-phase chromatography [21–25], chiral chromatography [26], and – by means of a simulation-based approach – nonlinear chromatography [10,27] to cite some of the most interesting cases.

This study focuses on a unified description of stochastic models through the so-called Lévy process representation or formalism [28,29], which has allowed for the establishment of a conceptual bridge between single-molecule dynamics observation and chromatographic experiments [30]. Paul Lévy (1886–1971) is universally recognized as the one "who has influenced the establishment and growth of probability theory more than any other." [31] He became a professor of analysis at École Polytechnique at Paris, France, in 1920, where he served until his retirement in 1959 [32]. Georges Guiochon (1931–2014) is remembered as one of the masters of modern chromatography and one of the students of Lévy, who graduated from École Polytechnique in 1951.

The continuous developments in analytical science and technology, which have decreased the limits of detection (in the order of femtomoles and yoctomoles [33,34]), have opened new,

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exciting frontiers of research and revived the stochastic models of chromatography. Indeed, experimental visualization of the erratic behavior of single molecules (e.g., by single-molecule fluorescence, optical tweezers, scanning probe microscopy, and mass spectrometry imaging [35]) has become feasible. These measurements can provide more information on the dynamic character of chromatography (in all of its fundamental aspects) than the ensemble-averaged data obtained from the classical analysis of a large number of molecules, as statistical fluctuations from the average lead to zone broadening [1,3,30].

The description of chromatographic separation as a Lévy process will be revised in the following section, with particular attention paid to its basic principles and physical meaning. Its applications and future possibilities of development will also be discussed.

2. Discussion

2.1. The microscopic stochastic model of chromatography

In the stochastic description of the chromatographic process, the chromatographic peak is measured by the probability density function of the time spent by the molecules inside the column. The retention time of a single molecule j is a random variable ($t_{R,j}$), which is calculated as the sum of all elementary times (τ) spent by that molecule in the mobile (M) and stationary (S) phases:

$$t_{R,j} = \sum_{i=1}^{n} \tau_{M,j,i} + \sum_{i=1}^{n} \tau_{S,j,i} \quad (1)$$

where n represents the random number of adsorption/desorption steps executed by the molecule during the chromatographic migration (with the index i representing the ith elementary step).

Again considering the separation process described in Fig. 1, where diffusion in the mobile phase is neglected, the number of adsorption events per unit time – also referred to as adsorption frequency (μ) – is related to the time t_M spent by an unretained molecule in the column. Indeed, in this case, the average number of adsorption events ($\bar{n}$) is constant for all molecules, and is given by

$$\bar{n} = t_M \mu = \frac{t_M}{\bar{\tau}_M} \quad (2)$$

where $\bar{\tau}_M$ is the average time spent in the mobile phase between two consecutive adsorptions (τ_M). In this case, Equation (1) reduces to

$$t_{R,j} = t_M + \sum_{i=1}^{n} \tau_{S,j,i} \quad (3)$$

It is well known that, in order to find the density function of a random variable, that is, the sum of two or more independent random variables, it is necessary to calculate the convolution integral of the density functions of the individual random variables. When the number of random variables becomes relevant, the convolution integral may become a very complex mathematical expression that could be analytically unsolved.

2.2. The Lévy model of chromatography

The problem of addition of random variables can be overcome by the use of a mathematical tool known as the *characteristic function* ($\varphi_t(\omega)$), that is, the Fourier–Stieltjes transform [36,37] of the frequency function in time domain. Then, the density function can be calculated by the inversion of the characteristic function obtained as the product of the characteristic functions of the individual random variables [38]. Furthermore, the statistical moments can be obtained directly from the derivatives of the characteristic function. The fundamental contribution to this field was made by Paul Lévy. In particular, he proved the central limit theorem using the characteristic functions and also introduced the concept of stochastic processes with independent and stationary increments. At present, these are commonly known as Lévy processes [39]. By means of the Lévy approach, it can be demonstrated that the chromatographic peak describing the model represented in Fig. 1 is given in the frequency domain by

$$\varphi_t(\omega) = \exp\left(i\omega t_M + t_M \int_0^{\infty} (e^{i\omega\tau_S} - 1) M(d\tau_S) \right) \quad (4a)$$

$$\varphi_t(\omega) = \exp\left(i\omega t_M + \sum_k (e^{i\omega\tau_{S,k}} - 1) \Delta M(\tau_{S,k}) \right), \quad (4b)$$

where i is the imaginary unit and $M(d\tau_S)$ (or in the discrete case $\Delta M(\tau_{S,k})$) is the differential of the Lévy "spectral function" $M(\tau_S)$. $M(d\tau_S)$ essentially represents the number of adsorption events with duration between τ_S and $\tau_S + d\tau_S$ per unit time. $M(\tau_S)$ can be obtained by the product of cumulative distribution function of the adsorption time, $F(\tau_S)$, and the adsorption frequency, μ, as

$$M(\tau_S) = \mu F(\tau_S), \quad (5a)$$

where the definition of adsorption time depends on whether desorption time is continuous ($f(\tau_S)$) or discrete ($f_j(\tau_{S,j})$):

$$F(\tau_S \le \tau_S^*) = \begin{cases} \int_0^{\tau_S^*} f(\tau_S) d\tau_S & \text{continuous} \\ \sum_j f_j(\tau_{S,j}) & \text{discrete} \end{cases} \quad (5b)$$

On the basis of the model used to describe the adsorption surface characteristics (e.g., homogeneity or heterogeneity), kinetics of the adsorption/desorption event (order of the reaction), and other attributes, the random variables in Equations (4) and (5) will be described by different probability distributions.

The physical meaning of the Lévy representation of the chromatographic process can be illustrated by considering a series of examples. Let us assume first a very simple case where the duration of any adsorption step is constant and equal to $\tau_{S,1}$ (with unit probability). Under this hypothesis, the spectral function $M(\tau_S)$ is a single Dirac delta function located at $\tau_{S,1}$, whose intensity μ will correspond to the number of molecules detected at that site per unit time (Equation 5). Let us assume furthermore that the number of transitions n between the mobile phase and stationary phase has a Poisson distribution, the most often used distribution to describe discrete stochastic processes (such as the number of adsorption events in chromatography). It designates the so-called memoryless processes, in which the probability that a given event occurs is only proportional to the observation length [4]. This simple model is represented in Fig. 2, whose bottom part shows the differential spectral function. The top part of the figure shows two different molecular trajectories followed by two molecules (A and B) injected simultaneously into the column (at (0,0)), which are presented by broken lines. Assuming that the time spent by either molecule in the mobile phase is constant, t_M, the total time spent in the stationary phase, and thus the total time spent in the column (t_R), will be clearly different, because of the randomness of the entry process. This randomness causes dispersion of arrival times at the column end, and thus peak broadening (even if the time τ_S is constant). The chromatographic peak in this simple case is represented as

$$\varphi_t(\omega) = \exp\left(i\omega t_M + t\mu \left(e^{i\omega\tau_{S,1}} - 1 \right) \right). \quad (6)$$

An adsorption surface paved by m different adsorption sites, each of which is characterized again by a constant adsorption time $\tau_{S,k}$ ($k = 1, \ldots, m$), is considered as the second example. $\tau_{S,k}$ depends on

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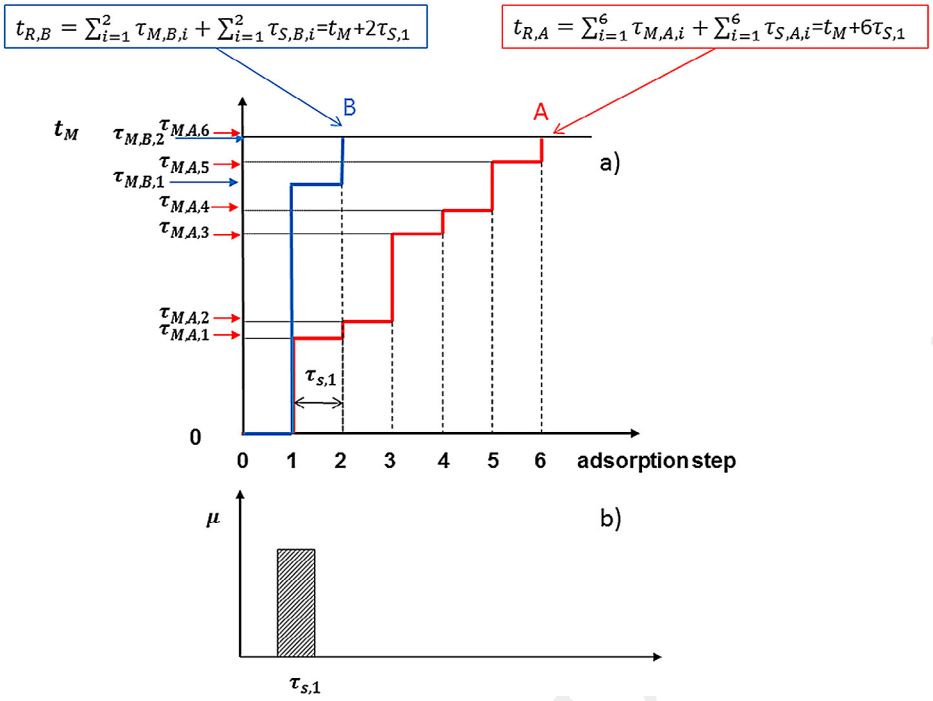

Fig. 2. (a) Molecular trajectories followed by two molecules (A and B) injected simultaneously into the column, at the point (0,0). The duration of any adsorption step is constant and equal to $\tau_{S,1}$; the number of adsorption steps follows a Poisson distribution. The time spent by both molecules in the mobile phase is constant and equal to t_M. (b) Differential spectral function of the process. The intensity μ represents the number of molecules detected per unit time by single-molecule experiments at the surface.

the adsorption energy of the kth site [28]; the larger the $\tau_{S,k}$ the stronger the adsorption site is [40]. The spectral function describing this case will be made by a series of Dirac delta functions located at $t_k = \tau_{S,k}$ with an amplitude μ_k, that is, the number of molecules detected at the kth site per unit time (or the abundance of kth-type site on the surface). For example, if $m = 4$, the differential spectral function will be similar to the distribution given in the bottom part of Fig. 3, where, in addition, it has been assumed that the number of molecules (per unit time) detected at the sites of types 1, 2, and 4 is equal and that twice as much molecules detected at site 3 ($\mu_1 = \mu_2 = \mu_4 = \mu_3/2$). In other words, sites 1, 2, and 4 are equally abundant on the surface, whereas the abundance of site 3 is twofold. In analogy with Fig. 2, the top part of Fig. 3 shows two random trajectories followed by two molecules (A and B). By assuming again that the time spent in the mobile phase is constant (t_M), the total time spent by the molecules in the column (t_R) will clearly depend on not only the number of adsorption steps (a random variable, n), but also the type of site visited in each adsorption step (being $\tau_{S,1} \neq \tau_{S,2} \neq \tau_{S,3} \neq \tau_{S,4}$). The corresponding representation of the chromatographic peak in frequency domain is

$$\varphi_t(\omega) = \exp\left(i\omega t_M + \frac{1}{\mu_t} \sum_{k=1}^{m} t\mu_k \left(e^{i\omega\tau_{S,k}} - 1 \right) \right), \quad (7)$$

where

$$\mu_t = \sum_{k=1}^{m} \mu_k. \quad (7a)$$

So far, we have considered cases where the random variables n and τ_s are described by discrete distributions. In the traditional Giddings–Eyring model (Fig. 1), the time spent by the molecule in the (homogeneous) stationary phase is considered as a random variable governed by exponential distribution (with average $\bar{\tau}_s$), whereas the number of adsorption/desorption steps follows a Poisson distribution (as in the discrete cases considered earlier). The resulting

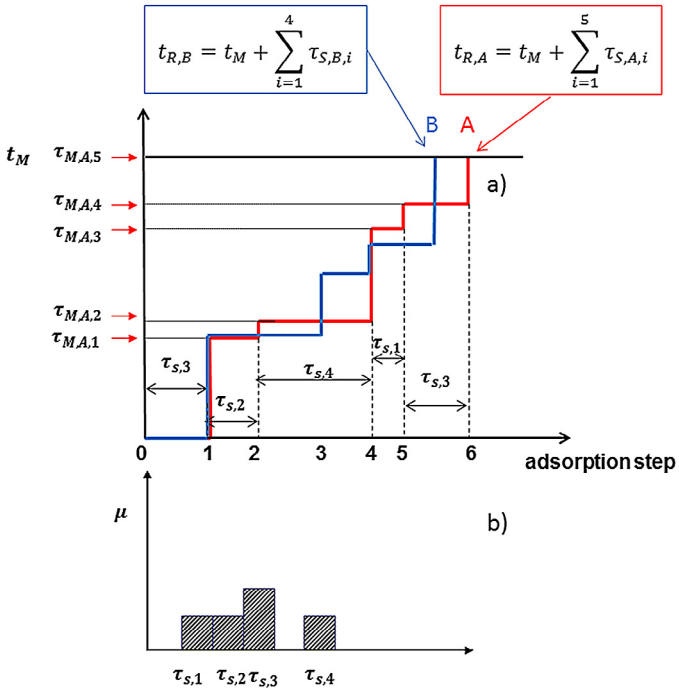

Fig. 3. The same as Fig. 2, but by assuming a heterogeneous surface made of four different adsorption sites. The duration of the adsorption events depends on the site where the molecule stops and may assume four different values ($\tau_{S,k}$ ($k = 1, \ldots, 4$)). (a) Molecular trajectories followed by two molecules (A and B). (b) Differential spectral function of the process. Intensities μ_k represent the number of molecules detected per unit time at the kth site (or the relative abundance of kth site on the surface). See text for details.

process can be classified as a *compound Poisson process*, and the chromatographic peak in the frequency domain takes the following form [9]:

$$\varphi_t(\omega) = \exp\left(i\omega t_M + \bar{n}\left[\frac{1}{1 - i\omega\bar{\tau}_s} - 1 \right] \right) \quad (8)$$

From a probabilistic viewpoint, it can be demonstrated that, if the frequency μ follows an exponential distribution and the number of adsorption sites (m) is sufficiently large [41], the discontinuous Lévy model of chromatography (Equation 4b) converges to the original Giddings–Eyring model.

However, the importance of the Lévy model for the interpretation of single-molecule experiments lies in its ability to deal with discrete distributions of residence times. Indeed, the experimental information that can be derived from single-molecule observation is usually in the form of number of molecules (or frequency) versus residence time [30] (Fig. 3). Therefore, by using the discrete Lévy representation, the experimental data can be used without any statistical–mathematical manipulation (e.g., nonlinear fitting procedures) to estimate average n or τ_s values based on a given model. In particular, for sparse events, additional manipulation may not only introduce bias in the estimation of the parameters, but also entail loss of information associated with rare events (e.g., adsorption on low-abundance sites). As it will be shown in the next section, important information about the molecular nature of the adsorption mechanisms has been deduced from the interpretation of these events. The major advantage of the Lévy representation over other microscopic stochastic models of chromatography is that it allows moving from an ensemble-averaged description of the process to the resolution of fundamental information at a single-molecular viewpoint.

2.3. *Insights into adsorption mechanisms by combining single-molecule observation and the stochastic theory of chromatography*

This section intends to provide a short overview of some of the most relevant results that have been achieved through single-molecule investigation and traditional chromatographic measurements for liquid–solid interfacial phenomena.

In one of the pioneering works where single-molecule spectroscopy has been used to investigate surface heterogeneity in reversed-phase liquid chromatography (RPLC), by studying the chromatographic behavior of a lipophilic cationic fluorescent dye on C_{18} stationary phase, Wirth et al. demonstrated the presence of two different "populations" of interactions [42]. Almost all molecules (~99%) diffused quickly at the solid–liquid interface (with a diffusion coefficient $D_s = 1.3\times10^{-6}$ cm^2/s), whereas about 1% of them was found to be stuck on the surface for a significantly longer time, in the order of several seconds. This behavior was successfully correlated to the presence of "strong" silanols on the surface by single-molecule imaging coupled to fluorescence correlation spectroscopy (FCS).

Later, those data were used to examine the applicability of the discrete Lévy model (Equation 7) to experimental data [28]. By considering a two-site heterogeneous adsorption surface made of 99% of weak sites and only 1% of strong ones, Pasti et al. demonstrated that the simulated peak obtained by the numerical inversion of Equation (7) was in good agreement with the experimental chromatograms provided in Ref. [43], as the time average is equivalent to the ensemble average, and the percentages of molecules found in Wirth's work correspond to the populations of the two types of sites on the surface.

In another interesting work, Wirth and coworkers indicated the presence of strong adsorption sites of topographical origin on the surface of C_{18} silica gel [44]. According to this work, which was using the same single-molecule techniques as in Ref. [42], the strong adsorption sites were considered equally important as acidic isolated silanols that explain the silanol activity of bonded silica phases. This information would have never been obtained without single-molecule measurements.

Recently, Mabry et al. [23] have studied the adsorption kinetics of a fluorescent lipid on a trimethylsilyl interface by combining single-molecule observations and ensemble-averaged data collected by RPLC experiments. By using the discrete Lévy representation of the chromatographic process (Equation 7), they were able to significantly interpret the dependence of peak tailing on the mobile-phase composition based on the correlation of the number of "active" strong sites (detected by single-molecule experiments) and the amount of organic modifier in aqueous organic eluents.

Further examples of combining single-molecule observations and chromatographic experiments have been provided by Landes et al. [45], who used superresolution single-molecule spectroscopy (SSMS) to study, with the aid of the stochastic theory, single-protein ion-exchange interactions on ligand-functionalized agarose stationary phases. This study demonstrated that, among the different ion exchangers considered, including pentaargininamide- and monoargininamide-functionalized and bare agarose, the protein was able to provide specific interactions solely on the pentaargininamide-based stationary phase. The adsorption/desorption times of single protein–ligand adsorption events were measured, through which a residence time versus frequency distribution (conceptually analogous to the one provided in the bottom part of Fig. 3) was obtained. Using the discrete Lévy model, the authors were able to reconstruct the chromatographic peak and provide an explanation, based on steric effect, for the heterogeneous single-protein adsorption/desorption kinetics observed with different molecules. In particular, they indicated that steric effect causes a reduction of interaction energy between protein surface and charged ligands, which leads to shorter desorption times and longer adsorption times, thereby causing peak fronting. In addition, they also compared the results of the discrete model with those obtained using the traditional Giddings–Eyring model (Equation 8), where the average number of adsorption steps ($\bar{n}$) and the average time spent in each step ($\bar{\tau}_s$) were obtained by fitting the experimental frequency distribution to an exponential function. The results clearly showed that only the discrete Lévy approach maintains the information contained in the experimental data, which, on the contrary, is irrecoverably lost by the ensemble-averaged representation.

The behavior of single proteins at ion-exchange interfaces was further investigated by Larson et al. [46], who, in particular, focused on the effect of ionic strength on these interactions. Their results support the hypothesis of spatial variations in adsorbent structural/energetic properties as a function of ionic strength to explain the observed heterogeneity of protein ion-exchange adsorption isotherms. They were also able to provide the first direct observation of mechanisms, leading to the narrowing of the functional-site population as ionic strength increases.

3. Conclusions

The combination of single-molecule observations and chromatographic experiments according to the representation of the discrete Lévy model represents a formidable tool for the investigation of interfacial processes of different nature. It is assumed that, in the near future, this approach will be particularly useful for the characterization and interpretation of biological systems, with the possibility offered by the model, in terms of not only revealing mechanisms of action at molecular viewpoint, but also by tracking the behavior of molecules in living cells [35].

It may reasonably be supposed that this methodology will contribute to the understanding of the differences between the molecular-scale environment and macroscopic world. In

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particular, it has been indicated [47] that there are two critical aspects where these differences become relevant: the Brownian motion and the relative importance of viscous forces over inertial forces. It has been shown that the actual motion of an object depends on not only the thermal forces exerted on the object, but also constraints (if any, e.g., viscous drag from the medium) on its motion. In order to gain a deep understanding of these aspects, single-molecule observations are needed in addition to the mean value of thermal energy. Inertial forces are dominant in macroscopic objects, whereas viscous drag force is more important than inertia at the microscopic level. This means that, at the microscopic scale, objects ~~do not move more rapidly than macroscopic ones if a force is not applied~~. The investigation of these features requires models that are capable of interpreting single-molecule behavior.

In conclusion, the major advantage of working with the raw, single-molecule data set is that far more information can be extracted from them than from ensemble-averaged distributions obtained from the classical analysis of a large number of molecules. Indeed, ensemble methods do not resolve the fundamental molecular viewpoint that reveals unique features of the process under investigation.

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