Single-Molecule Spectroscopy

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Single-Particle and Ensemble Diffusivities—Test of Ergodicity**

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Diffusion is the omnipresent, random motion of matter, such as atoms and molecules, driven by thermal energy and is the key for innumerable processes in nature and technology.[1] In nearly every chemical reaction diffusion is the key mechanism of bringing the reactants in close proximity, which is an essential prerequisite before any reaction can take place. Additionally many reactions are diffusion-controlled, meaning that the reaction kinetics is limited by the diffusion process. Central to the dynamics of diffusion, and in general matter, is the ergodic theorem,[2] which states that for systems in the equilibrium state the time average taken over a single particle is the same as the ensemble average over many particles. However, while being generally accepted no experimental validation has so far been reported. Here, we present experimental proof of this fundamental theorem by measuring under identical conditions the diffusivities of guest molecules inside a nanostructured porous glass using two conceptually different approaches. The data obtained through the direct observation of dye molecule diffusion by single-molecule tracking experiments,[3] that is, the time-average, is in perfect agreement with the ensemble value obtained in pulsed-field gradient NMR experiments.[4]

After one and a half centuries of diffusion measurements with large ensembles of diffusing particles,[5] the option of single-particle tracking (SPT) with single-molecule sensitivity has recently provided us with a totally new view of diffusion. In this approach, the trajectory of a single, optically labeled molecule can be recorded during a sufficiently long interval of time. The obtained trajectory can thereafter be analyzed to access, for example, the average value of the squared displacement $r^2(t)$ of a diffusing particle during a time interval t [Eq. (1)].

$$\langle r^2(t)\rangle_{\text{time}} = \lim_{T\to\infty}\frac{1}{T-t}\int_0^{T-t}(r(\tau+t)-r(\tau))^2 d\tau \tag{1}$$

Exactly this quantity is in the most straightforward way measured using the diffusion gradient NMR technique. Here, however, the mean square displacements measured are averaged taken over about 10^{20} diffusing species [Eq. (2)]:

$$\langle r^2(t)\rangle_{\text{ensemble}} = \iint_{r,r_0}(r-r_0)^2 p(r_0)P(r,t;r_0)\,dr\,dr_0 \tag{2}$$

where $p(r_0)$ and $P(r,t;r_0)$ denote, respectively, the ("a priori") probability that a molecule is found at position r_0 within the sample and the ("conditional") probability that, after time t, a molecule has moved from r_0 to r. For both r_0 and r, the integration extends over the whole sample space.

The direct comparison of these two quantities obtained for one and the same system may yield essential information on microscopic mechanisms of mass transfer in systems exhibiting deviations from normal diffusion including out-of-equilibrium situations[6] and, more generally, ergodicity breaking.[7] However this is an extremely difficult experimental problem. Even the seemingly simple case of equilibrium systems, forming the basis for the proof of the ergodic theorem, so far remained unregarded in the literature.

To date, the mutually contradicting measuring conditions have prohibited the application of ensemble and single-particle techniques to one and the same system: The trajectory of a diffusing single molecule is constructed by fitting the position of the molecule over time with SPT.[3a] Therefore the fluorescence signals of the molecules have to be clearly separated from each other, which requires very low concentrations. Additionally the measurements are limited by the signal-to-noise ratio, which is influenced by the brightness of the dye molecules as well as the integration time. Consequently there is an upper limit for the detectable diffusivity in SPT. Exactly the opposite conditions, namely high concentrations (for generating sufficiently strong signal intensities) and high diffusivities (for giving rise to observable displacements) must be fulfilled for the application of the pulsed-field gradient (PFG) technique of NMR spectroscopy, representing the most powerful ensemble technique for diffusion studies.

Bridging the gap between SPT and ensemble measurement did thus require a thoughtful selection of both the probe molecule and the host system. Among a large variety of

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fluorescing molecules, Atto532 (ATTO532-COOH, ATTO-TEC, Siegen, Germany) dissolved in deuterated methanol offered particularly favorable properties for both techniques, namely a long transverse nuclear magnetic relaxation time for PFG NMR spectroscopy (see the Methods Section in the Supporting Information) and a sufficient photostability and quantum yield for single-molecule spectroscopy. Both single-particle observation[8] and PFG NMR ensemble studies[9] have revealed porous glasses as a most versatile host system for diffusion studies. Moreover, with the option of a continuous variation of the mean pore diameter[10] porous glasses offer the unique option to "adjust" the guest diffusivities to those values where the sensitivity ranges of SPT and ensemble measurements overlap.

So far, the application of porous glasses as a standard material for diffusion measurements in nanopores was limited by the fact that the lower limit of controlled pore sizes in monolithic materials has been on the order of 4 nm, as a consequence of uncontrolled phase separation in the sodium borosilicate initial glass melt.[10] This limitation has now been overcome by a modified roller-quenching process[11] in combination with an optical fine cooling[12] so that the fabrication of nanoporous glasses with a homogeneous pore surface and pore diameters down to 1 nm have now become possible.[13] Here we used such glasses with a random three-dimensional pore structure and pore sizes of 3 nm as determined from nitrogen adsorption at 77 K (see the Supporting Information).

First, the diffusion properties of dye molecules in the nanoporous host were studied using gradient NMR spectroscopy. To solely detect the NMR signal of the dye molecules under study, we have chosen deuterated methanol as a solvent and tuned the working NMR frequency of the spectrometer to that of protons residing on the dye. The NMR samples (see the Methods section in the Supporting Information) contained both solutions within the pores as well as some excess bulk phase. Thus, there existed two populations of the dye molecules with different diffusion properties, with faster diffusivities in the bulk and with slower diffusivities in the pore system due to confinements,[9] with the relative weights determined by dynamic equilibrium between the two "phases". Consequently, the primary quantity measured, namely the NMR spin-echo diffusion attenuation Ψ, had been contributed by both ensembles [Eq. (3)]:[4b,14]

$$\Psi(q,t) = p_{\mathrm{pore}}\exp(-q^2tD) + (1-p_{\mathrm{pore}})\exp(-q^2tD_{\mathrm{bulk}}) \qquad (3)$$

In Equation (3), q is the wave number externally controlled in the experiments, p_{pore} is the relative fraction of dye molecules in the pores, D_{bulk} and D are the diffusivities of the dye molecules in the bulk solution and in the solution within the pores. Notably, the use of the sum of two exponential functions, uncoupled from one another, in Equation (3) is fully justified by the fact that molecular exchange between the two ensembles during the diffusion times of the order of tens of milliseconds used in our experiments was negligibly small because of the macroscopic extension of the porous monolith.[14]

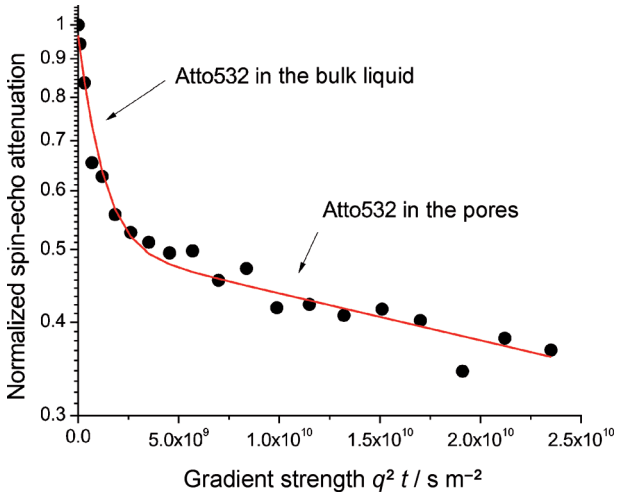

Figure 1. PFG NMR spin-echo diffusion attenuation of a dye ensemble. Data are fitted with a bi-exponential decay thus accounting for diffusion inside the pores and in excess medium.

Figure 1 shows a typical PFG NMR spin-echo diffusion attenuation of a dye ensemble with the solid line being the fit of Equation (3) to the experimental data. Notably, the experiments performed with varying diffusion time t yielded the diffusivities which, in the considered interval from 5 to 100 ms, did not depend on the observation time, thus revealing normal diffusion. Exactly the thus obtained diffusivities D are further shown in Figure 3.

The diffusion of dye molecules in the glass material was studied additionally by single-molecule fluorescence microscopy (see the Methods section in the Supporting Information). By collecting several fluorescence images of the single molecules using wide-field microscopy and determining the position of the molecules in each image, single-molecule trajectories are obtained (an example can be seen in Figure 2a.) The diffusion coefficient for each single-molecule trajectory can be extracted from the linear part of the mean square displacement (MSD) plots according to $\langle r^2(t)\rangle = 4Dt$ assuming an isotropic Brownian diffusion in all three dimensions and keeping in mind that the fluorescence images correspond to a two-dimensional projection of the three-dimensional diffusion. An isotropic Brownian motion is justified by the fact that the particle diffuses in a three-dimensional pore structure und the displacements followed in the experiments exceed the pore diameters by orders of magnitude. To test whether diffusion is dependent on the dye concentration at low filling ratios, we performed experiments at increasing concentrations keeping in mind that the signal separation of different single dye molecules and out-of-plane fluorescence becomes limiting at higher concentrations. The MSDs of 170 single Atto532 molecules from samples with concentrations of 3.2×10^{-11} mol L^{-1}, 3.2×10^{-10} mol L^{-1}, and 6.4×10^{-10} mol L^{-1} were measured. As an example the MSD plots of 70 single molecules of a concentration of 3.2×10^{-11} mol L^{-1} are shown in Figure 2b. The MSD plots fit well to other examples of single-molecule diffusion.[15] Additionally the cumulative distribution of the logarithm of the single-molecule diffusivities of this sample is depicted in

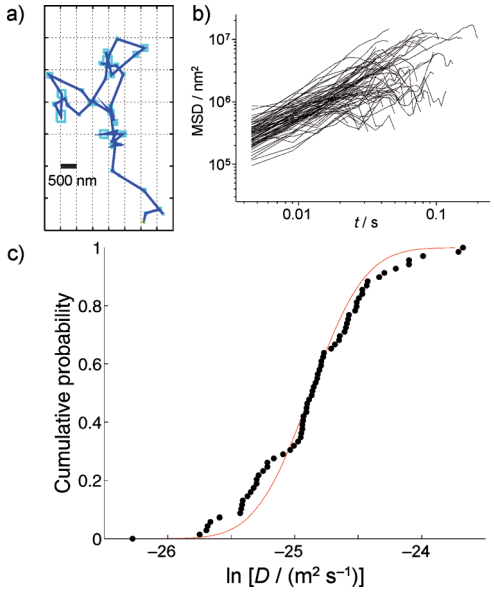

Figure 2. Single-molecule studies of dye molecule diffusion in nanoporous glasses. a) Typical trajectory of a single dye molecule diffusing in the porous host system. For each time point the experimentally determined positioning accuracy is depicted by box-error bars. b) MSD plots obtained from the analysis of 70 single-molecule trajectories of Atto532 dye molecules. The measurement was performed using a dye concentration of 3.2×10^{-11} mol L^{-1}. c) Cumulative distribution of the logarithm of the single-molecule diffusivities of the sample with a dye concentration of 3.2×10^{-11} mol L^{-1}. The data are fitted assuming a log-normal distribution using a maximum likelihood estimation (red line).

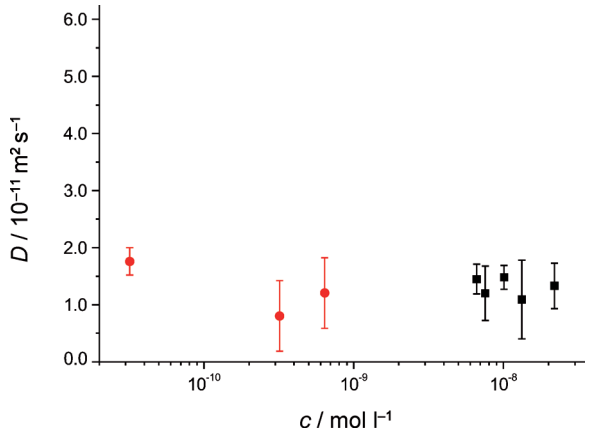

Figure 3. Mean diffusivities (D) of Atto532 molecules inside the porous host system (pore size 3 nm; c = concentration). Single-molecule (red circles) and PFG NMR spectroscopic (black squares) mean values.

Figure 2c. Both distributions show the heterogeneity of diffusion for different molecules, which is hidden to ensemble measurements due to averaging. The data are fitted to a log-normal distribution using a maximum likelihood estimation (red line).

To compare the data obtained from PFG NMR spectroscopy und SPT, the mean diffusivities of Atto532 molecules inside the porous host system are shown in Figure 3. The data plotted as red circles correspond to the mean values of the observed distributions of single-molecule diffusion coefficients. The error bars are computed keeping in mind tracking and statistical errors as well as sample-to-sample variations and the lower signal-to-noise ratio for higher guest molecule concentrations. Mean values obtained by PFG NMR spectroscopy are shown as black squares. Irrespective of the fact that the measuring conditions may thus be adjusted to allow the application of both techniques to one and the same host–guest system, the maximum guest concentrations in SPT turned out to remain separated from the minimum concentrations in PFG NMR spectroscopy by still one order of magnitude. Even under such conditions, however, the measurements may become fully comparable if molecular diffusion is controlled by host–guest interactions, with the host surface being sufficiently homogeneous for ensuring a host–guest interaction that is independent of the guest concentration.

Single-molecule and ensemble diffusion measurements are found to experimentally confirm the hypothesis of ergodicity for the first time because (within the limits of accuracy) both techniques provide the same result (Figure 3). With these experiments, the two so far separated worlds of diffusion measurements have been brought together. As a prerequisite of this combination we have considered a situation where the rules of normal diffusion are obeyed. However, single particle observations of, for example, biological systems[15,16] often seem to contradict ergodicity. In many studies of this kind, the mean square displacement $\langle r^2(t)\rangle$ is found to deviate from the "normal" dependence $\langle r^2(t)\rangle \propto t$,[6,17] with the mean square displacement generally increasing less than linearly with the observation time. Among the reasons leading to such subdiffusive dynamics, "macromolecular crowding" and "obstacle effects"[17] are considered as the most probable and decisive ones. Under these conditions ergodicity breaking, that is, the difference between the messages of SPT and PFG NMR spectroscopy, might occur for example because of aging effects. They correlate with the broad distribution of the mean residence times of the particles in the subvolumes of the system and with its variation during the evolution of the system. Now, with the combined potentials of single particle and ensemble measurements, we lay out the basis for future studies aiming at the clarification of the possible conditions and underlying reasons for the resulting patterns of ergodicity breaking.

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