UNCOVERING NON-ERGODICITY ON THE CELL MEMBRANE USING SINGLE PARTICLE TRACKING APPROACHES

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Abstract. In this work, we study the diffusion on the plasma membrane of the receptor DC-SIGN. The data we used were obtained by Single Particle Tracking technique and hence consist of individual trajectories. Motivated by investigating the dynamics of this receptor, our analysis comprises not only of standard statistical approaches but also of examining ergodicity. For the wild type receptor and a mutant, the diffusion process was found to be non-ergodic. In order to test our analysis and conclusions, we also generated simulations for Brownian and confined random walkers.

Keywords: Diffusion, Ergodicity, Single Particle Tracking, Receptor Dynamics, DC-SIGN

1. Introduction

The plasma membrane is a very complex environment that consists of proteins, receptors and lipids. This heterogeneous environment, overcrowded with proteins that might exhibit spatial organization at different scales and/or interaction with the cytoskeleton or other membrane components, affects protein diffusion [1]. Understanding the dynamics and the organization of proteins on the cell membrane is crucial for better comprehension of cellular functions such as signal transduction, pathogen recognition, endocytic transport and cell-cell adhesion [2].

Recent advances in optical and fluorescence microscopy have sharpened experimentalists’ tools to unravel these mechanisms. Among these techniques, Single Particle Tracking (SPT) constitutes a powerful tool for unraveling membrane proteins’ dynamics at the single molecule level. SPT, is based on the detection of individual molecules tagged with fluorescent markers [3]. The sample is illuminated in epifluorescence or Total Internal Reflection Fluorescence (TIRF) geometry with excitation light of a specific wavelength, which is absorbed by the marker. Upon excitation the markers emit light at longer wavelengths which, after proper filtering is collected by a CCD camera. Typically, experiments are performed at low labeling conditions, resulting in a few fluorescent markers contemporarily imaged on the CCD field of view [4]. In this way, even though the images are diffraction limited, the markers’ fluorescent images are spatially well separated, allowing to determine their centroid positions with high accuracy (20-30 nm) [4]. Videos are collected at high temporal resolution (10- 500 frames/s). Automated algorithms allow to identify and then reconnect the markers’ positions frame by frame, providing the trajectories of single proteins diffusing on the plasma membrane [4].

SPT and the inherent trajectory analysis based on mean square displacement (MSD) has been extensively used to characterize the motion of several membrane components [5, 6]. These works have shown that membrane components might undergo different types of motion, namely free
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Brownian diffusion, subdiffusion and confined motion [5], reflecting different interactions with membrane lipids, actin cytoskeleton or other proteins. Recently, a new analysis method, based on the comparison of temporal and ensemble average of particle displacements, have been applied to SPT data of ion channels [2] showing that they can undergo non-ergodic diffusive behavior on the cell membrane. In this work, we applied both the standard and the new analysis method to thoroughly characterize the diffusion of the pathogen-binding receptor DC-SIGN and two of its mutants. In addition, simulations of single molecule trajectories performed according to different types of diffusion were generated and analyzed to validate our analyses.

1.1 Motivation
DC-SIGN (Dendritic cell-specific ICAM-3-grabbing nonintegrin) is a type II membrane C-type lectin receptor that is present on both macrophages and dendritic cells. It binds to various microorganisms and functions as a receptor for several viruses and pathogens (HIV-1, ebola virus, hepatits C virus, Candida albicans, Mycobacterium tuberculosis) [7]. DC-SIGN is a transmembrane protein that consists of an extracellular and a transmembrane part followed by a cytoplasmic tail that contains recycling and internalization motifs. The extracellular part comprises a carbohydrate recognition domain (CRD), which forms the head region and a 7.5 tandem repeat of 23 aminoacids that forms the neck region [8]. It has been shown that DC-SIGN organization is related to the cell function [7, 8]. More specifically, previous works suggested that the receptor is spatially organized in nanoscale domains on the plasma membrane [9] and this favors virus binding and internalization in dendritic cells [7]. Apart from that, it has been also shown that different mutants of this receptor (N80A-DC-SIGN, lacking the glycosylation motif and ΔRep-DC-SIGN, lacking the neck region) show differences in its spatial organization, resulting in more efficient binding to nanometer sized pathogens and also determine the internalization efficiency of the pathogens [8]. Since the plasma membrane is a fluid environment in which receptors undergo different types of diffusion, reflecting the interactions with other membrane components, we decided to investigate if, besides the spatial organization at the resting state, the DC-SIGN dynamics also had a role in determining the cellular function.

Besides standard analysis of single particle trajectories, Weigel et al. [2] have recently proposed another formalism based on temporal and ensemble average showing that ergodic and non-ergodic processes coexist in the cell membrane. They studied the physical mechanism behind the subdiffusion of Kv2.1 potassium channels on the cell membrane of mammalian cells and they actually linked the dynamical organization of this membrane protein to the function of the cell. The Ergodic Hypothesis states that a system is ergodic when the time average value of an observable, which is determined by the dynamics, is equal to an ensemble average that is an average over one time for a large number of observables which all have identical properties but are not identical on the molecular level [11]. This essentially means that in an ergodic system the averages of a given observable quantity taken over time and space can be exchanged [10]. In the case in which the temporal and the ensemble averages are not equal, the system is non ergodic and thus the long term evolution of such a system cannot be quantified neither we can predict its behavior in an average. In the case of single particle trajectories belonging to several DC-SIGN molecules diffusing on the cell membrane, the observable quantity corresponds to the square displacement at a given time lag.

1.2 Methodology
In this work we used trajectories of the wild type DC-SIGN (wt-DC-SIGN) and two mutants, N80A-DC-SIGN and ΔRep-DC-SIGN, obtained by deleting specific regions of the wt-DC-SIGN receptor that were transfected in Chinese Hamster Ovary (CHO) cells. As fluorescent markers, we used quantum dots. The quantum dots used for the SPT were streptavindin-coated quantum dots (Qdot655, Invitrogen). The samples were imaged with a custom single molecule
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epifluorescence microscope. The data-set we used consisted of 727 trajectories, up to 2000 frames long, for the wt-DC-SIGN, 614 trajectories up to 1200 frames long for the N80A-DC-SIGN mutant and 101 trajectories up to 632 frames long, for the ΔRep-DC-SIGN mutant. The frame rate in the three cases was 60 frames per second. An example of three trajectories of the data set that contains the wt-DC-SIGN is shown in figure 3.A. All the analysis was performed using custom routines written in Matlab (The MathWorks).

Figure 1: (A) An example of three trajectories from the wt-DC-SIGN data set. (B) MSD versus time lag for 26 trajectories of wt-DC-SIGN.

In the standard analysis of the particle trajectories it is common to use the Mean Square Displacement (MSD) [5, 12], which allows a characterization of the diffusion mode and estimation of the diffusion coefficient.

For this kind of analysis, first we need to find the Square Displacements (SDs) of the trajectories. The SDs, $R^2$, are calculated from the coordinates X and Y, for all lag times:

$$R^2_{m,ij} = (X_{i+m,j} - X_{i,j})^2 + (Y_{i+m,j} - Y_{i,j})^2$$  \hspace{1cm} (1)

where $i$ is the frame number, $j$ is the trajectory number, $m$ is the lag time, $t_{lag}=m/t_1$ and $t_1$ is the frame rate [2]. In this way we store the SDs of each trajectory on a matrix and each line corresponds to a different lag time. To be more specific, on the first line, the first element corresponds to the SD of the particle between the first and second frame, while the second element corresponds to the SD between the second and third frame. On the second line, the first element corresponds to the SD of the particle between the first and third frame, and so on. The MSD is a measure of the average distance that a particle travels and is calculated for each trajectory. It is defined as:

$$\text{MSD}(m, i, j) = \langle (X_{i+m,j} - X_{i,j})^2 + (Y_{i+m,j} - Y_{i,j})^2 \rangle$$ \hspace{1cm} (2)

where $(X_{i+m,j} - X_{i,j})^2 + (Y_{i+m,j} - Y_{i,j})^2$ is the SD of the particle $j$, that is averaged over a time interval of length $m+i$. Examples of MSD versus time lag for the wt-DC-SIGN, is shown in figure 1.B.

In the case of Brownian motion, the molecule moves from one point of the membrane to another and Einstein showed that the square distance grows linearly with time [12]. Thus, for a Brownian particle diffusing in two dimensions $\text{MSD}=4Dt$, where $D$ is the apparent diffusion coefficient, meaning the magnitude of the diffusion of the molecule on the cell membrane [5, 12]. Hence, from the MSD we can calculate the apparent diffusion coefficient. On the other hand, in the case of anomalous diffusion the MSD scales sublinearly with time, $\text{MSD}=t^\gamma$, where $\gamma$ is the characteristic anomaly exponent and if $\gamma>1$ the motion is superdiffusive whereas if $\gamma<1$, the
motion is subdiffusive. [5, 12] For the third mode of motion, the confined motion, the MSD reaches a plateau with time, according to the equation:

$$\text{MSD} \equiv \langle r^2 \rangle_c \left[1 - A_1 \exp\left(-\frac{4A_2Dt}{\langle r^2 \rangle_c}\right)\right] \quad (3)$$

where $\langle r^2 \rangle, A_1, A_2$ are constants determined by the geometry of the confined motion [5]. In the case the motion is confined on a square lattice, the lattice size is $L = \sqrt{3} \cdot r$ (4) [5].

Another common formalism that gives information about the diffusion of the particles, consists in the use of the cumulative distribution function (CDF) of particle displacements [13, 14]. The CDF gives the probability that a particle which is placed at the origin at time zero, will be found within a circle of radius $r$, at time $t$. For a particle that has a fast and a slow mobility components with $D_1$ and $D_2$ diffusion coefficients respectively, and $w$ and $(1-w)$ fractions respectively, the particle’s CDF fits to a bi-exponential curve of the form:

$$\text{CDF}(r^2, t) = 1 - w \cdot \exp\left(-\frac{r^2}{r_1^2}\right) + (1 - w) \cdot \exp\left(-\frac{r^2}{r_2^2}\right) \quad (5)$$

where $r_1$ and $r_2$ are defined by the diffusion constant of the fast and slow mobility components respectively, $r_1^2 = 4D_1t_{\text{lag}}$ [13,14]. For Brownian motion $w$ is close to one (or $1-w$ close to zero) and the CDF becomes mono-exponential, whereas for a motion with two mobility components the value of $w$ is significantly smaller than one.

Single particle trajectory can alternatively be analyzed by comparing the distributions of the temporal and ensemble MSD [2], determining the ergodicity of the system under observation. The temporal MSD is the mean squared distance that a particle has travelled within a specific time i.e. is calculated for the total length of the trajectory. Thus, the distribution of the temporal MSD of all the trajectories is a measure of the distance travelled by the particles within a long period of time. The ensemble MSD is the square distance that all the particles have travelled, but for a very small time interval i.e. is calculated for one frame. In this way, we get a measure of the distance that all the particles have travelled, at a very short period of time. If both distributions are equal, we can then get a safe result concerning the dynamics of the system either by measuring for extended period of time the motion of the particles, or by just following the particles for a very short period of time, since the diffusion process is ergodic. The temporal MSD of a specific time lag is found if we average over the frames $i$, for each trajectory $j$. So for each trajectory the temporal MSD is:

$$\langle \Delta r^2(t) \rangle_T = \frac{1}{M} \sum_{i=1}^{T/t_1} R_{m,i}^2 \quad (6)$$

where $M$ is the total number of SDs and $T$ is the total averaging time [2].

The ensemble MSD is found if we average over the trajectories $j$, at each frame $i$:

$$\langle \Delta r^2(t) \rangle_{\text{ens}} = \frac{1}{N} \sum_{j=1}^{N} R_{m,i}^2 \quad (7)$$

where $N$ is the total number of trajectories at frame $i$ [2].

Finally, we analyzed our data through the temporal-ensemble MSD (TEA-MSD). The calculation of the TEA-MSD was done by applying an additional average to the temporal MSD [2]. To be more specific, if we store all the SDs of each trajectory at a specific lag time, in the resulting matrix each line corresponds to one trajectory and each column to the SD of the trajectory at each frame. In other words, on this matrix, the first line has the SDs of the first trajectory for a specific lag time and thus in the first element is stored the SD between the first and second frame (if we suppose that we calculate for the first lag time, $t_{\text{lag}} = 1 \cdot t_1$, where $t_1$ is the frame rate), the second
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element consists of the SD between the second and third frame and so on. The TEA-MSD is found by averaging over the frames for all the trajectories, meaning that the first point on the resulting graph consists of the mean value for the first frame for all the trajectories, then the second point for the first and second frame, the third for the first, second and third frame, and this averaging continues for the total time T. Thus the TEA-MSD for a total time T at $t_{lag} = m \cdot t_1$ is:

$$\langle \langle (\Delta r^2)_{T} \rangle \rangle_{ens} = \frac{t_1}{N \cdot T} \sum_{i}^{T} \sum_{j}^{N} R_{m,i,j}^2$$  \hspace{1cm} (8)$$

The TEA-MSD for anomalous diffusion scales as a power law, for all lag times, of the form:

$$\langle \langle (\Delta r^2)_{T} \rangle \rangle_{ens} \sim T^{\alpha-1}, \text{ with } \alpha -1<1.$$  

2. Results

We started analyzing the data set which contained the trajectories of wt-DC-SIGN, N80A- DC-SIGN and ΔRep-DC-SIGN by calculating the apparent diffusion coefficient in each case. This was done by generating the MSD in an ensemble average as a function of lag time. For every lag time we calculated the mean value of the square displacement for every trajectory and then we averaged over the trajectories, at every lag time (figure 2.A-C). In all cases we truncated the experimental data so that all the trajectories have the same length, 100 frames.

![Figure 2](image-url)

Figure 2: Error bar plots of the mean square displacement (MSD) in an ensemble average versus time lag for wt-DC-SIGN (A), N80A-DC-SIGN (B) and ΔRep-DC-SIGN (C). The plots can be fitted well by a power law curve of the form MSD = k \cdot t^\alpha. For wt-DC-SIGN k=0.29±0.01 μm^2/s^\alpha and α=0.94±0.01, for N80A-DC-SIGN k=0.13±0.01 μm^2/s^\alpha, α=0.91±0.01 and for ΔRep-DC-SIGN k=0.55±0.01 μm^2/s^\alpha and α=1.03±0.01 (errors are standard errors). (D-F) Square displacement CDFs of all the data sets in semilog plots. The solid black lines represent the mono-exponential fitting and the solid red lines represent the double exponential fitting. wt-DC-SIGN (D) can be fitted better by the bi-exponential curve with $w=0.36$, and similarly $w=0.47$ for N80A-DC-SIGN (E) and $w=0.31$ for ΔRep-DC-SIGN (F).

From the plots of figure 2, we found that in all cases, the data were fitted well at a curve of the form MSD = k \cdot t^\alpha, as described in the figure’s caption. Therefore, according to the equation MSD=4Dt we found the apparent diffusion coefficient that shows the degree of the mobility of the particles. For wt-DC-SIGN we found $D = 7.1 \cdot 10^{-2} \mu m^2/s$, for N80A-DC-SIGN $D = 3.33 \cdot 10^{-2} \mu m^2/s$ and for ΔRep-DC-SIGN the diffusion coefficient was found with a significantly larger value of $1.6 \cdot 10^{-1} \mu m^2/s$. To further investigate our findings we generated the CDFs of
all trajectories at many lag times and fit the data to mono and bi-exponential curves. In figure 2.D-F we show the resulting graphs for $t_{lag} = 1 \cdot t_1$. Apparently, the CDFs of wt-DC-SIGN, N80A-DC-SIGN and ΔRep-DC-SIGN fit better to the bi-exponential curves than to the mono-exponential ones, suggesting that although the MSD analysis implies Brownian diffusion, there is a heterogeneity on the diffusion coefficient.

Figure 3: Mean Square Displacement versus time lag on a log-log plot, of 26 representative trajectories, for wt-DC-SIGN (A), N80A-DC-SIGN (B) and for ΔRep-DC-SIGN (C). (D-F) Distributions of temporal MSD and (G-I) distributions of ensemble MSD. For wt-DC-SIGN, the temporal distribution (D) is broader than the ensemble one (G), for N80A-DC-SIGN the temporal distribution (E) is a lot broader than the ensemble (E), while for ΔRep-DC-SIGN the temporal (F) is comparable to the ensemble (I).

In figure 3.A-C we show the MSD versus time lag, of some representative trajectories, for all the data sets. The next analysis step was to generate the temporal and ensemble MSD distributions. Both averages were generated for equally long trajectories of 100 frames, for all the trajectories that includes each data set. We again truncated the trajectories so as they all have the same length, 100 frames. Figure 3 displays the resulting distributions, where for wt-DC-SIGN (figure 3.D, G) and N80A-DC-SIGN (figure 3.E, H) the temporal MSD distribution is much broader than the ensemble one. On the contrary in the case of ΔRep-DC-SIGN (figure 3.F, I), the distributions are still not equal (for equal temporal and ensemble MSD distributions, the process is ergodic) but the temporal MSD is comparable to the ensemble. We then examined the TEA-MSD for all the data and at five different time lags. Figure 4 (A-C) shows the TEA-MSD versus the total averaging time $T$, calculated for lag times 1, 3, 5, 10 and 30. In all cases the TEA-MSD fits well to a power law of the form $\langle \Delta r^2 \rangle_T \sim T^{\alpha-1}$, with $\alpha$-1 being slightly smaller than unity. We also plotted the MSDs for total times $T=6s$ and $T=9.5s$, versus $t_{lag}$ (figure 4.D-F). From this plot we see that
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The TEA-MSD again scales as \( \langle (\Delta r^2)_{ens} \rangle \sim t_\text{lag}^\gamma \) with \( \gamma \) being slightly smaller than unity for the three data sets. The data points in figure 4.D-F for both total averaging times \( T \) almost overlap, and graphically is difficult to observe a difference.

![Figure 4: Log-log plots of the time-ensemble MSD versus total averaging time \( T \) of wt-DC-SIGN (A), N80A-DC-SIGN (B) and \( \Delta \text{Rep}-\text{DC-SIGN} \) (C). The straight lines indicate the power law fit. In all cases the slope is \( a_1<1 \). For wt-DC-SIGN \( a_1=0.97\pm0.01 \), for N80A-DC-SIGN \( a_1=0.95\pm0.01 \) and for \( \Delta \text{Rep}-\text{DC-SIGN} \) \( a_1=0.95\pm0.01 \) (errors are standard errors). The lower panel plots (D-F) are the MSDs against \( t_\text{lag} \), for total times \( T=6 \) s (black circles) and \( T=9.5 \) s (red circles) of wt-DC-SIGN, N80A-DC-SIGN and \( \Delta \text{Rep}-\text{DC-SIGN} \) (D, E, F respectively). The data points for both total times almost overlap and graphically is difficult to observe any difference. In both total times and in all data sets, the MSDs scale exponentially with the critical exponent being less than 1, but again very close to unity. For wt-DC-SIGN the exponent \( \gamma=0.90 \) for both \( T=6 \) s and \( T=9.5 \) s. Similarly, for N80A-DC-SIGN \( \gamma=0.92 \) and for \( \Delta \text{Rep}-\text{DC-SIGN} \) \( \gamma=0.93 \).

In order to assess our previous analysis and algorithms, we simulated Brownian and confined random walkers. The first simulation is the Brownian motion for which we simulated up to 1000 particles, each one starting from a random point, with diffusion coefficient 0.08 \( \mu m^2/s \). Then each walker jumps to one of its nearest locations randomly with a time step of 16ms for a total number up to 1000 frames. In addition, we simulated confined movement, by placing the walker at the center of a squared lattice and by restricting its motion to the boundaries of this square. This was done by applying reflecting boundary conditions on the square lattice. We simulated the confined random walkers for three different sizes of this square lattice, 1000nm, 400nm and 100nm respectively.

An example of one trajectory in each case of the simulations is shown in figure 5.A. In the case of the Brownian motion, the simulated particle, moves randomly in space whereas in the case of the simulated confined motion we see that the particle is restricted to the boundaries that we set. Furthermore we constructed the MSDs versus time lag, for 500 trajectories, 100 frames long each (figure 5. B, C). In the case of Brownian motion the MSD fits perfect to the equation \( \text{MSD}=4Dt \) and thus scales linearly with time (figure 5.B), and the diffusion coefficient is found to be indeed 0.08 \( \mu m^2/s \). On the other hand for all the confined simulations we see that the MSDs reach a plateau and fit perfectly at the equation for the confined motion (equation (3)) (figure 5.C). The smaller the size of the square lattice we simulated, the less time is needed for the MSD to reach...
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the plateau. This is because the random walker needs less time to explore the square box compared to the larger sized square boxes. From the MSD fitting, we found that for the 1000nm box \( r_c = 0.33 \, \mu \text{m}^2 \), for the 400nm box \( r_c = 0.053 \, \mu \text{m}^2 \) and for the 100nm box \( r_c = 0.0033 \, \mu \text{m}^2 \). According to calculations using the equation (4) we indeed find that the square lattices we simulated are 1000nm, 400nm and 100nm. The above show that there is a consistency between what we wanted to simulate and the result.

According to calculations using the equation (4) we indeed find that the square lattices we simulated are 1000nm, 400nm and 100nm. The above show that there is a consistency between what we wanted to simulate and the result.

Figure 5: (A) A particle’s trajectory in space, in the Brownian simulation the trajectory moves freely whereas in the confined simulation the particle is restricted to the boundaries that we set in each case. (B-C) The Mean Square Displacement in an ensemble average as a function of time. The MSD scales linearly in the case of the Brownian simulation (B). The MSD for the confined simulations reaches a plateau (C). The smaller the square lattice at which the particles are restricted, the sooner the MSD reaches the plateau.

Since all our simulations represent random processes, we expect to see ergodic behavior, meaning the distributions of the temporal and ensemble MSDs should be equal in all the simulations. Ergodicity can be inspected though only if we follow an observable for enough period of time and only if we follow enough observables. Thus a question, of how many trajectories and how long they should be in order to detect ergodicity in random processes, is raised. For that, in each simulation we tested upon what restrictions ergodicity is indeed observed. We generated simulations for multiple number of particles and also with multiple number of trajectory lengths, for Brownian and confined motions. The simulations clearly show that only for a minimum of 500 particles with trajectory length of 100 frames each, the temporal MSDs are equal to the ensemble ones (figure 6.A-D). This is not the case for less number of particles (100) even if the trajectory length is as high as 1000 frames (figure 6.E). Thus, we see, that the above two variables -the number of particles analyzed as well as their trajectory length- are the most crucial in our initial task. In other words, in order to be confident determining whether a motion is ergodic or non ergodic, we must have experimental data that fit the above criterion.
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3. Conclusions

In this work we analyzed three data sets that include the trajectories from wt-DC-SIGN, N80A-DC-SIGN and ΔRep-DC-SIGN. We calculated the diffusion coefficients, the MSDs in an ensemble average and the CDFs for all the trajectories in each data set. This part was the standard analysis, from which we found that ΔRep-DC-SIGN is diffusing significantly faster than wt-DC-SIGN and N80A-DC-SIGN. Although the standard analysis shows a behavior consistent with Brownian diffusion, from the CDF analysis we found that the CDFs in all data sets we used fit better to a bi-exponential curve, with the weighting factor w being significantly less than unity. This indicates heterogeneity in the motion of the different particles of each receptor type. The TEA-MSD analysis, confirms this behavior by showing a power law dependence with the critical exponents being close to yet smaller than unity, in the three cases.

The simulations of Brownian and confined random walkers, showed that in order to have trustful conclusions regarding ergodicity, the data sets we analyze must contain at least 500 trajectories and each trajectory has to be at least 100 frames long. In the case of wt-DC-SIGN and N80A-DC-SIGN, those two criteria are reached. Therefore, we can confidently say that in these two cases the diffusion process on the cell membrane is non-ergodic, since the distribution of the temporal

Figure 6: Temporal (upper panel) and ensemble (lower panel) Mean Square Displacement of the: Brownian simulation (A), confined to 1000nm box motion (B), confined to 400nm box motion (C), confined to 100nm box motion (D). The averages were obtained for 500 trajectories with 100 frames length each. In all cases both distributions are equal. The same averages for Brownian simulation of 100 particles, 1000 frames each (E). In this last case, the widths of the distributions are not equal.
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MSD is not equal to the distribution of the ensemble MSD. On the other hand, in the case of ΔRep-DC-SIGN the data set we used contains only 101 trajectories. Even though all the trajectories are longer than 100 frames, still the first criterion, meaning the number of trajectories, is not reached. In this case, the distributions of the temporal and ensemble MSD are not equal but comparable, and this indicates that the diffusion process in this case might be ergodic.

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