Diffusion through Nanopores in Connected Lipid Bilayer Networks

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A biomimetic model of cell-cell communication was developed to probe the passive molecular transport across ion channels inserted in synthetic lipid bilayers formed between contacting droplets arranged in a linear array. Diffusion of a fluorescent probe across the array was measured for different pore concentrations. The diffusion characteristic timescale is found to vary nonlinearly with the pore concentration. Our measurements are successfully modeled by a continuous time random walk description whose waiting time is the first exit time from a droplet through a cluster of pores. The size of the cluster of pores is found to increase with their concentration. Our results provide a direct link between the mesoscopic permeation properties and the microscopic characteristics of the pores, such as their number, size, and spatial arrangement.

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In multicellular organisms, cell-cell communication is essential for morphogenesis, cell growth, and differentiation as well as cell homeostasis [1]. Cells have thus developed various mechanisms to communicate with each other, such as the release of solutes or vesicles in their environment, electrical signals, and direct cell-cell contacts. Within direct cell-cell contacts, communication through molecular exchange is made possible with protein gates that create nanopores spanning between apposed cytoplasmic cell membranes. Plants and fungi for instance, use, respectively, the so-called plasmodesmata and septal pores. In animals, two kinds of pores, gap junction channels, and tunneling nanotubes have also been identified and are very similar in their structure and function to their plants and fungi counterparts. Gap junctions, in particular, consist of juxtaposed protein-based hemichannels that can assemble into clustered structures of typical size a few hundreds of nanometers [2]. They enable a passive diffusion-based transport of small hydrophilic molecules between connected cells, whose properties are mostly measured for cells in vitro with dye transfer techniques [3], such as gap fluorescence recovery after photobleaching [4], for instance, allowing one to determine the permeability of the gap junction.

In recent years, the use of well controlled artificial multicellular systems to design complex reaction-diffusion processes within the framework of bottom-up synthetic biology has considerably increased. In particular, synthetic membranes such as droplet interface bilayers (DIBs) [5], which are obtained by putting in contact aqueous droplets bathing in an oil-lipid mixture, have allowed for the study of molecular transport through both passive ion channels [6,7] and active transporters [8] using fluorescence imaging. Very recently, networks of DIBs connected by passive staphylococcal α-hemolysin (αHL) pores were used to probe genetically engineered reaction-diffusion-based processes [9]. In all of these experiments, both for cells in vitro and for artificial systems, diffusion processes across either gap junctions or DIBs decorated with nanopores are usually modeled with Fick’s law combined with a phenomenological permeation law through a membrane, yielding a large scale effective diffusion coefficient. The microscopic mechanisms that underly the permeation law and therefore control the value of this effective diffusion coefficient are, however, poorly described. In particular, its dependence with the concentration of the nanopores in the membrane and their spatial arrangement has never been evidenced experimentally, or modeled theoretically.

In this Letter, we report on a thorough study of the diffusion of molecular probes through linear networks of aqueous droplets connected by DIBs decorated with αHL pores at different concentrations. We model the diffusion from one droplet to its neighbors with a continuous time random walk model whose waiting time is the first exit time from a droplet, either through independent pores or through clusters of pores. Our experimental measurements strongly suggest that the diffusion law is controlled by the clustering of the nanopores and provide estimates of the clusters size as a function of αHL’s concentration.

Monomers of αHL (Sigma-Aldrich) were diluted in an aqueous buffer [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10 mM, KCl 100 mM, Sigma-Aldrich, pH 7.4] at concentrations c ranging from 150 to 300 μg/ml. Diphantanol-θ-glycero-3-phosphocholine lipids in chloroform (4ME 16:0 PC, Avanti Polar Lipids) were evaporated under nitrogen and resuspended
at a concentration of 6.5 mg/ml in a mixture (50:50 vol:vol) of hexadecane and silicone oil AR20 (Sigma-Aldrich).

Droplets of the αHL solution were produced inside a Plexiglas pool decorated with parallel grooves and form a DIB network. Lateral positioning of the droplets is achieved by moving the pool with a motorized translation stage. Eventually, a source droplet seeded with fluorophores is deposited. Diffusion of fluorophores is imaged in epifluorescence microscopy. (Inset) Image of three typical DIB networks. The white bar is 200 μm long. (b) Sketch of the diffusion process of fluorophores across DIBs decorated with αHL protein pores.

was a signature of a DIB formation [see the pairs of white segments at each contact on Fig. 2(a)]. In practice, the resulting networks had from three up to ten droplets (see the Supplemental Material [11]).

Source droplets containing a solution of fluorophores (5-carboxyfluorescein from Sigma 20 μM in HEPES 10 mM, KCl 100 mM) were then added either at the end [Fig. 2(a)] or in the middle of a DIB array (see the Supplemental Material [11]). To limit evaporation of the aqueous droplets, a coverslip was placed on the oil-lipid pool [Fig. 1(b)]. Diffusion of fluorophores from the source droplet to its neighbors through the nanopores was imaged in epifluorescence microscopy overnight (typically for 15 h, every 11 min). A typical diffusion process over 16 h is shown in Fig. 2. Control experiments (see the Supplemental Material [11]) in the absence of αHL nanopores were also performed, and no significant increase of the fluorescence intensity in the neighboring droplets was measured in 90% of the cases. However, in some cases, an osmotic shock could occur, create transient pores in the DIB, and lead to a rapid increase of the fluorescence signal in immediate neighbors over short timescales (typically less than 1 h). Such fast kinetics
events can be easily identified and have also been excluded from our analysis.

To quantify the diffusion process, image analysis was performed on bright field images in order to find the radii and center coordinates of each droplet using a homemade MATLAB routine. The fluorescence intensity $I_i(t)$ inside each droplet $N_i$ was measured by taking the average intensity inside a disk that measures half the total droplet size. We checked, however, that the value of $I_i(t)$ does not change with the disk size. Furthermore, we have verified that the measured fluorescence intensity is proportional to the concentration of fluorophores in the explored concentration range. The occupancy probability $P_i$ inside a droplet (except for the source droplet) was then defined as

$$P_i(t) = \frac{I_i(t) - I_i(t_0)}{I_i(t) + \sum_{j \neq i}[I_j(t) - I_j(t_0)]}, \quad (1)$$

where $I_i(t_0)$ is the average intensity at the beginning of the experiment and $I_i(t)$ the intensity of the source droplet, and where the summation runs over all droplets in the network but the source. For the source droplet, the numerator of Eq. (1) is taken as $I_i(t)$ so that $P_i(t_0) = 1$. Therefore, $P_i(t)$ decreases with $t$ as fluorophores diffuse from the source to its neighbors, while $P_i(t)$ of the neighboring droplets $N_i, j \neq i$ increases [Fig. 2(b)]. Note that two possible artifacts can affect the time evolution of the fluorescence intensities $I_i(t)$ and $I_i(t)$. On the one hand, a size decrease of the droplets due to evaporation can cause an increase of the fluorophore concentration and thus of the fluorescence intensity. Photobleaching, on the other hand, can cause a decrease of the measured fluorescence intensity. To quantify these combined effects, we measured on control experiments (without any pores, and thus without any diffusion away from the source) the time variation of $I_i(t)$. We found that the source intensity increases moderately over time (about 10% of relative increase in 13 h; not shown). We can conclude that evaporation effects dominate over photobleaching. We further checked that the definition of $P_i(t)$ was efficient in correcting this artifact. For the control experiment [Fig. 2(b), black disks] the value of $P$ is indeed equal to unity over the whole time course of the experiment. Last, considering experiments with pores, we excluded from our analysis the rare experiments for which the evaporation rate was anomalously high and dominant over diffusion, yielding an increase of the source intensity $I_i$ over time.

We studied the diffusion kinetics by specifically focusing on the source and first neighbor droplets and using different networks with different $\alpha$HL monomer concentrations. The introduced monomers $\alpha$HL$_m$ are first adsorbed and then diffuse within the bilayer to form a heptamer $\alpha$HL. Since the heptamerization process is fast [12], we describe this chemical sequence as a single step equilibrium $7 \alpha$HL$_m$ ↔ $\alpha$HL, which implies that the number of pores adsorbed inside the bilayer scales with the monomer concentration $c$ as $c^7$.

Figure 3(a) shows the occupancy probability $P_1$ for the first neighbor as a function of time $t$ for increasing $\alpha$HL monomer concentrations $c = 150, 200, 250, 300 \mu g/ml$, with $N = 18, 25, 8, 10$, respectively. Error bars indicate the standard deviation of the data. The dashed lines are linear fits used to extract the characteristic rate $\lambda$ (see the main text). They are shifted vertically to ease visualization. (b) Characteristic rate $\lambda$ as a function of $c$. The solid line is a power-law fit of exponent $3 \pm 1$. The dashed line is a fit using an exponent of $7/2$. The dashed-dotted line is a fit using an exponent of $7$. (Inset) Occupancy probability as a function of the rescaled time $(\lambda) t$. 

![Diagram showing the occupancy probability as a function of time and concentration](image-url)
Theoretically, for an infinite array of connected compartments, one can model the time evolution of $P_i$ using a continuous time random walk approach [13]. Each probe molecule is described as a random walker jumping to the adjacent site with a time-dependent probability. The corresponding waiting time is defined as the first exit time of a molecule from a droplet. For 3D Brownian diffusion, the latter is known to be exponentially distributed with a mean value that we denote as $\tau$ [14]. Since diffusion within a droplet occurs on timescales much shorter ($\sim 10$ s, with a diffusion coefficient of carboxyfluorescein in water $D = 4 \times 10^{-10}$ m$^2$/s [15]) than the typical diffusion time from one droplet to its neighbors ($\sim 1$ h), one can assume that the fluorophore concentration is uniform within the droplets and therefore can model the droplet network with a set of discrete connected sites. Within this framework, one can derive using standard tools [13] the occupancy probability within the first neighbor $P_i^{th}$

$$P_i^{th}(t) = e^{-t/\tau} I_1(t/\tau),$$

where $I_1$ is the modified Bessel function of the first kind. In our experiments, droplet arrays have a finite size. Thus, Eq. (2) cannot model our data at long times. However, in the short time limit ($t/\tau \ll 1$), Eq. (2) yields to first order $P_i^{th} \sim \lambda t$ with $\lambda = 2/\tau$, independently of the total number and position of the droplets.

Experimentally, at very short times, our data show a small deviation from linearity [Fig. 3(a)] that we interpret as the combined result of both DIB equilibration and protein adsorption kinetics at the lipid bilayer. Past this regime, one observes that $P_i(t)$ is approximately linear. For the highest concentration of nanopores, $P_i(t)$ eventually reaches a steady state. For all experimental curves used to compute the average, we have identified this linear regime and chosen to fit it to extract $\lambda$. This was done by testing for the existence of an inflection point at $t = t_{in}$ and extracting the slope $\lambda$ in its vicinity. In case the inflection point was nonexistent, we fitted the last 3 h of the data. Whatever the method used, we checked that the values of $\lambda$ were equally distributed within experimental error bars. For the purpose of illustration, we have performed linear fits on the averaged $P_i(t)$, as shown with the dashed lines in Fig. 3(a). We also linearly fitted every single curve to extract $\lambda$ as a function of the nanopore concentration. Resulting $\lambda$’s are shown in Fig. 3(b) on a log-log plot and are consistent with a power-law dependence with $c$. Fitting this data with a power-law yields $\lambda \sim c^{3 \pm 1}$. The value of this exponent will be discussed farther down theoretically.

The inset of Fig. 3(b) shows the occupancy probability as a function of the rescaled time $t/\tau$ where $\langle \tau \rangle$ is the average value plotted on the main panel. All curves at different $c$ collapse on the same master curve, indicating that $\lambda^{-1}$ is the only timescale that governs the diffusion kinetics. In recent years, DIBs have increasingly been used [9,16] to study both passive and active transport of molecules using fluorescence-based measurements [6,7,9]. All of these studies assume that diffusion of a molecular probe across a membrane can be described with a phenomenological permeation law, from which an effective large scale permeation coefficient is deduced. However, the microscopic origins that set its value have not been explored. Within the theoretical description presented above [see Eq. (2)], we propose that the molecular transport from one droplet to another is fully characterized by the mean waiting time $\tau$ in a droplet. This is the average time necessary for a chemical messenger to reach any single pore of diameter $a$ within the cell, i.e., the first exit time [17,18]. For a confining domain of volume $V$ and a single pore, this time has been obtained theoretically [17–19] and is written in the large $V$ limit

$$\langle T_1 \rangle = \frac{V}{2Da}.$$ For a spherical domain of radius $R$, this time is much larger—by a factor $R/a$ ($\sim 10^5$)—than the typical time needed for a probe to explore in bulk a typical length $R$. Importantly, this result was shown to be independent of the shape of the confining volume. In addition, it has been shown for 3D diffusion in Refs. [20,21] that in the case where $d \gg a$, where $d$ is the distance between targets, pores can be considered independent. As a result, the first passage time $\langle T_n \rangle$ simply is written $\langle T_n \rangle = \langle T_1 \rangle/n$ independently of both the shape of the confinement domain and the spatial organization of targets. When $d \sim a$, $T_n$ is still independent of the shape of the confining domain, but screening effects [20,21] between targets have to be taken into account. In the limit of a close-packed configuration, i.e., if $n$ pores are clustered, they can be considered as a single unit of area $na^2$, with a typical size $a' = \sqrt{na}$, and thus $\langle T_n \rangle = \langle T_1 \rangle/\sqrt{n}$.

Since aHL is a heptamer, we expect for both cases the characteristic diffusion rate $\lambda$ to scale either as $c^2$ if pores are considered independent, or as $c^{7/2}$ if pores are clustered. Shown in Fig. 3(b) (dashed line) is a power-law fit of $\lambda(a)$ with an exponent 7/2, in reasonable agreement with the data. On the contrary, a power law with an exponent 7 is far from providing a quantitative agreement. It suggests that the diffusion kinetics are controlled by clustered rather than independent nanopores. This nonlinearity of the diffusion characteristic time contrasts with previously used phenomenological models [9]. Using expressions of $\langle T_n \rangle$, one can also deduce the typical cluster sizes $\sqrt{na} \approx \lambda \langle T_1 \rangle a$. Taking $R = 75$ μm, $D = 4 \times 10^{-10}$ m$^2$/s, and $a = 1.4$ nm [22] gives sizes of clusters that range from about 10 nm ($n \approx 100$) at the lowest concentration to about 100 nm ($n \approx 10^4$) at the highest concentration. This clustering should also depend on membrane composition, which
might explain the discrepancies in the reported αHL pore concentrations between different lipid mixtures [23]. Interestingly, it has recently been shown theoretically in Ref. [24] that stochastic variations of transmittance between compartments lead to anomalous diffusion in networks of compartments. In our experiments, the distribution of sizes of the nanopore clusters could induce randomness of the transmittance and explain the variability of our data.

We have used DIBs decorated with αHL passive ion channels to mimic the passive molecular transport through biological cells. Using fluorescence imaging, we have quantified the effect of the pore concentration on the diffusion kinetics of a molecule from one cell to its neighbors. We have found that the diffusion kinetics are efficiently captured by a continuous time random walk model. We have also found that the characteristic diffusion timescale varies as the inverse square root of the number of pores within the lipid bilayer, suggesting a pore clustering scenario. Such clustering has been evidenced numerically for transmembrane proteins [25]. Taking into account this clustering for nanopores is thus likely to be relevant for molecular transport in real biological systems.

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