Monte Carlo simulations of protein micropatterning in biomembranes: effects of immobile nanofeatures with reduced diffusivity

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Abstract

Nanoscopic features of reduced diffusivity have long been suggested to contribute to plasma membrane heterogeneity. Two prominent examples of this are highly dynamic lipid-mediated assemblies (‘membrane rafts’) and shells of annular lipids surrounding transmembrane proteins. Here, we simulated a micropatterning experiment, where such nanoscopic features are immobilized in specific areas within the live cell plasma membrane. We evaluated the effect of patterned nanofeatures of different sizes and diffusivities on the spatial distribution and two-dimensional mobility of tracer molecules. From this, we derive empirical models that describe the long-range tracer mobility as a function of the nanofeature density. In turn, our results facilitate the determination of nanofeature dimensions from micropatterning experiments.

Supplementary material for this article is available online

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(Some figures may appear in colour only in the online journal)

1. Introduction

The perception of the plasma membrane has changed over the last decades from a fluid, rather homogeneous environment for lipids and proteins [1] towards a micro- and nanostructured matrix that controls cellular processes via the formation of spatial lipid/protein heterogeneities [2, 3]. Various mechanisms were suggested to contribute to this compartmentalization, including the immobilization of membrane proteins via the cortical cytoskeleton [4] or exoskeleton fences [5], protein-protein interactions [6], lipid phase separation [7], or curvature-mediated formation of protein superstructures [8], to name a few.

Besides organizing the plasma membrane scaffold per se, non-homogeneous protein distribution further affects the mobility and spatial distribution of most other membrane constituents by defining diffusional barriers [9]; prominent examples include tight junctions in epithelia [10], and the axonal initial segment [11] as well as the synaptic membranes in neuronal cells [12]. In such protein meshes, the mobility—and hence permeability—becomes massively reduced for protein clusters compared to monomers [13], making such areas efficient size filters. Of note, transmembrane proteins are known to associate with an annular ring of lipids on a
nanoscale [14, 15]. Up to a hundred lipids may co-diffuse with the transmembrane protein forming a ring of reduced mobility extending several nanometers from the protein surface [16].

We have previously introduced live cell protein micropatterning as a tool to study the effective size of plasma membrane constituents—lipids as well as proteins—by determining the retardation of a fluorescently labelled tracer diffusing through regions of deliberately enriched and immobilized proteins [17, 18]. In this approach, target proteins are rearranged in the live cell plasma membrane by growing cells on surfaces featuring micropatterns of antibodies. Micropatterned surfaces can be readily produced using soft lithography methods such as microcontact printing, reaching spatial resolutions down length scales of a few tens of nanometers [19], which allows for deliberate reorganization and enrichment of the target proteins to ON-regions, leaving OFF-regions depleted of target protein (figure 1(A)). Single particle tracking (SPT) of a fluorescently labelled tracer molecule can then be used to investigate tracer distribution and mobility in dependence of the nanofeature density.

In order to be able to extract target protein size as well as tracer-target interactions from diffusion data, we have previously employed extensive Monte Carlo simulations of tracers diffusing through regions of immobilized obstacles [20]. Here, we extended our previous study by addressing the effects of immobilized nanofeatures with reduced diffusivity, or lipid shells with reduced diffusivity co-immobilized with the target proteins (figure 1(B)). We derived mobility ratios constants \(D_{\text{ON}}/D_{\text{OFF}}\) and tracer densities \(\rho_{\text{ON}}/\rho_{\text{OFF}}\) in ON- versus OFF-regions and provide guidelines for the quantitative interpretation of such micropatterning experiments.

2. Methods

2.1. Simulation of nanofeatures

All simulations were performed in MATLAB (R2019a, The MathWorks Inc. Natick, MA) using a standard personal computer. Random walks of non-interacting point tracers were simulated on two-dimensional square maps containing random distributions of nanofeatures within the ON-region. The diffusion constant outside of the nanofeatures was set to \(D_{\text{out}}\) (see table 1 for a list of variables). We simulated two different types of nanofeatures: (i) circles with diffusion constant \(D_{\text{in}} < D_{\text{out}}\) mimicking immobilized lipid rafts of radius \(R\), (ii) circular rings (radius \(R\)) with diffusion constant \(D_{\text{in}} < D_{\text{out}}\) surrounding single central impermeable circular obstacles (radius \(R_{\text{core}}\)) mimicking immobilized transmembrane proteins including a ring of associated lipids (figure 1(B)). In the following, index in indicates nanofeature segments of low diffusivity, index out membrane segments of high diffusivity (indicated in light grey and white in figure 1(B), respectively).

2.2. Implementation of the micropattern geometry

We considered here a micropatterning experiment, in which circular regions of immobilized nanofeatures are generated (termed ON-region) within regions devoid of such nanofeatures (OFF-region) (figure 1(A)). In a real experiment, the size of the ON-region is on the order of a few micrometers, and hence more than hundred-fold larger than the immobilized nanofeatures. As output parameters of the simulation, we analyzed the ratio of tracer surface densities \(\rho_{\text{ON}}/\rho_{\text{OFF}}\) and the mobility ratio \(D_{\text{ON}}/D_{\text{OFF}}\). For determination of \(\rho_{\text{ON}}/\rho_{\text{OFF}}\), we simulated a single circular ON-region within a square OFF-region of identical area (dashed box in figure 1(A)). Note that in the absence of nanofeatures by definition the diffusion constant remains unaffected \(D_{\text{OFF}} = D_{\text{out}}\); for determination of \(D_{\text{ON}}/D_{\text{OFF}}\) it was hence sufficient to simulate the diffusion behavior in ON-regions.

2.3. Distribution of nanofeatures

Nanofeatures were distributed with uniform probability in the ON-region. Overlapping of individual circular nanofeatures was permitted, yielding arbitrarily shaped patterns. In some cases, however, we were interested in the effects of clustered distributions of nanofeatures. To this end, nanofeatures with center coordinates \((x, y)\) were positioned on randomly generated probability maps defined by \(p(x, y) \propto \sum_{i=1}^{N_C} \exp \left(-\frac{(x-x_i)^2+(y-y_i)^2}{2\sigma^2}\right)\), where the number of clusters is denoted by \(N_C\), the uniformly distributed cluster centers by \((x_i, y_i)\), and the cluster size by \(\sigma\). The density of nanofeatures was measured in terms of number density \(\rho\) or area fraction \(C\). For practical reasons, we defined an intended area fraction \(C_{\text{intended}}\), which was used to calculate the simulated density of nanofeatures according to [21]

\[
C_{\text{intended}} = 1 - e^{-\rho \pi R^2}.
\]  

Since this equation is only valid for uniformly distributed nanofeatures, we finally quantified the effectively covered area fraction \(C\) by creating pixelated masks with pixel size of \(0.1 \times 0.1 \text{ nm}^2\), and counting pixels covered by the simulated nanofeatures. This was particularly relevant in case of clustered distributions of nanofeatures, where \(C \neq 1 - \exp(-\rho \pi R^2)\) due to heterogeneously distributed nanofeatures.

We calculated the total area fraction characterized by diffusion constant \(D_{\text{in}}\) as \(C_{\text{in}}\). Additionally, we set \(C_{\text{in}}\) in relation to the area fraction accessible to the tracer molecules \(1 - C_{\text{core}}\): we termed this ratio \(C_{\text{in}}/C_{\text{core}}\). For simulation of type 1 nanofeatures mimicking lipid rafts, \(C_{\text{in}} = C_{\text{in}} = C\). In contrast, type 2 nanofeatures mimicking transmembrane proteins also contain an impermeable obstacle core with area fraction

\[
C_{\text{core}} = 1 - \exp\left(-\rho_0 \pi R_{\text{core}}^2\right),
\]  

\(C_{\text{core}}\) relates to the total area fraction \(C\) via \(C_{\text{core}} = 1 - (1 - C_{\text{in}})R_{\text{core}}^2/R^2\). The ratio \(C_{\text{in}}\) is hence given by \(C_{\text{in}} = \frac{C - C_{\text{core}}}{1 - C_{\text{core}}} = 1 - \left(1 - C\right) \left(R^2 - R_{\text{core}}^2\right)/R^2\) or in terms of number density by \(C_{\text{in}} = 1 - \exp\left(-\rho_0 \cdot (R^2 - R_{\text{core}}^2)\right)\).
2.4. Simulation of tracer diffusion

We defined all lengths of our simulation in units of the nanofeature radius $R$. Diffusing tracer molecules were represented by point tracers with equal partitioning probability for membrane segments characterized by $D_{in}$ and $D_{out}$. For all simulations we assumed periodic boundary conditions. At the beginning of each simulation, point tracers were distributed uniformly in the accessible space and started their random walk without mutual interactions. Off-lattice random walks were realized by repeatedly displacing tracers by a fixed step length $l$ in a random direction, where $l$ was chosen dependent on whether the tracer was located in a segment with $D_{in}$, with $D_{out}$, or crossing between the two. For each tracer position we determined whether the tracer was inside or outside of a nanofeature by calculating the distance $d$ to the closest nanofeature center and comparing it to the nanofeature radius $R$. For $d < R$ or $d > R$ a tracer was considered inside or outside, respectively. Crossing was detected by a change in the tracer environment from inside to outside or vice versa.

In order to adequately describe the transition between membrane segments of different diffusion constants, we assumed an underlying ballistic transport model. Briefly, the microscopic diffusion process can be interpreted as the result of a sub-nanoscopic ballistic motion in conjunction with elastic collisions on randomly distributed hypothetical particles. Hence, at this length scale $l$ can be interpreted as the free path length of the tracer’s ballistic motion. The density of these hypothetical particles defines $l$ as well as the time for a single step $\Delta t$. The step length and step time reflect the underlying sub-nanoscopic velocity $v = \sqrt{D/\tau}$, yielding the microscopic diffusion constant $D = \frac{v^2}{\Delta t} = \frac{l^2}{\Delta t}$. Importantly, in this model the transition between segments of different diffusion constants is described without a change in velocity, but instead via a change in the density of the hypothetical particles. Hence, step lengths and times of two segments with different diffusion constants are related as $\frac{D_{in}}{D_{out}} = \frac{l_{in}}{l_{out}} = \frac{\Delta t_{out}}{\Delta t_{in}}$. Naturally, $l_{out}$ happens to represent the longest step length in the simulation, and was set to $l_{out} \leq R$.

For tracers crossing from $D_{out}$ to $D_{in}$, both the step length $l_{cross}$ and step time $\Delta t_{cross}$ have to be modified. For this, we calculated the fraction $f$ of a single step the tracer would have spent in the new environment, assuming no alteration of the tracer mobility at the border. Next, we resized step length and step time in order to account for the altered tracer mobility in the new environment, yielding $l_{cross} = (1 - f) \cdot l_{out} + f \cdot l_{in}$ and $\Delta t_{cross} = (1 - f) \cdot \Delta t_{out} + f \cdot \Delta t_{in}$ (figure 2(A)). Crossing in the opposite direction was implemented analogously. Tracers detected inside a nanofeature core (i.e. $d < R_{core}$) were repositioned according to their ballistic reflection from the core surface.

In some cases, we were interested in the effects of spontaneously forming rafts. To this end, we employed a probabilistic trapping model [22]. Freely diffusing tracers were assumed to switch their mobility between $D_{out}$ and $D_{in}$ randomly in time, with characteristic transition time constants $\tau_{out}$ and $\tau_{in}$, respectively. $\tau_{out}$ and $\tau_{in}$ were chosen to reflect average residence times as observed in simulations with immobilized nanofeatures. The time fraction $\beta = \frac{\tau_{in}}{\tau_{out} + \tau_{in}}$ directly corresponds to the area fraction $C$ covered by type 1 nanofeatures.

2.5. Analysis of simulated trajectories

In a real life SPT experiment, the described sub-nanoscopic motion of the tracers is not accessible. Instead, a typical experiment yields a sequence of positions of the tracer separated by time intervals $t_{delay} \gg \Delta t$. We assumed here stroboscopic illumination with illumination times short enough to virtually freeze the motion of the particle during exposure. To
mimic this experimental situation, we analyzed simulated trajectories by interrogating tracer positions $\tilde{r}(t)$ at regular time intervals $t_{\text{delay}}$ (figure 2(B)). Note that the differences between time steps $\Delta l_{\text{in}}$ and $\Delta l_{\text{out}}$ as well as the resizing of step times $\Delta t_{\text{cross}}$ introduced small shifts in the discrete time basis for the different trajectories, preventing a precise definition of $t_{\text{delay}}$. For convenience, we defined a set-point for $t_{\text{delay}}$, selected the closest actual time-points within the trajectories, and took the average.

As common in SPT, square displacements were calculated via $r^2(t_{\text{lag}}) = \left[ \tilde{r}(t + t_{\text{lag}}) - \tilde{r}(t) \right]^2$ for a large range of time lags with $t_{\text{lag}} = n \cdot t_{\text{delay}}$. Mean square displacements were calculated as $\text{msd}(t_{\text{lag}}) = r^2(t_{\text{lag}})$. Throughout this manuscript, we calculated the diffusion constant $D_{\text{ON}}$ by analysis of the mean square displacement as a function of the time lag $t_{\text{lag}}$ according to $D_{\text{ON}} = \frac{\text{msd}}{4t_{\text{lag}}}$.

Plotting the decadic logarithm of the mobility ratio $D_{\text{ON}}/D_{\text{OFF}}$ as a function of $\log_{10}(t_{\text{lag}})$ resulted in sigmoidal curves (see Results). The curves were approximated with the sigmoidal function

$$\log_{10}(D_{\text{ON}}/D_{\text{OFF}})(t_{\text{lag}}) = -\frac{1}{2} \left( a_1 \cdot a_2 \sqrt{\pi} \right) \times \text{erf} \left( \frac{1}{a_2} \cdot (a_3 - \log_{10}(t_{\text{lag}})) \right) + a_4$$

with erf denoting the error function $\text{erf}(x) = \frac{2}{\sqrt{\pi}} \int_0^x \exp(-t^2) dt$, and $a_1$, $a_2$, $a_3$, $a_4$ the free fit parameters. In this representation, physically interesting quantities can be derived from the fit parameters, such as the mobility ratio at low time lags,

$$D'_0 = \lim_{t_{\text{lag}} \to 0} D_{\text{ON}}/D_{\text{OFF}} = 10^{-\frac{1}{2}} a_1 \cdot a_2 \sqrt{\pi} + a_4$$

and the mobility ratio at large time lags

$$D'_\infty = \lim_{t_{\text{lag}} \to \infty} D_{\text{ON}}/D_{\text{OFF}} = 10^{\frac{1}{2}} a_1 \cdot a_2 \sqrt{\pi} + a_4.$$ (5)

We observed anomalous subdiffusion in the ON-regions with $\text{msd}_{\text{ON}} \propto t_{\text{lag}}^\alpha$, yielding a proportionality $D_{\text{ON}}/D_{\text{OFF}} = \frac{\text{msd}_{\text{ON}}}{\text{msd}_{\text{OFF}}} \propto t_{\text{lag}}^{-\alpha}$. In a double-logarithmic plot this proportionality translates to $\log_{10}(D_{\text{ON}}/D_{\text{OFF}}) \propto (\alpha - 1) \cdot \log_{10}(t_{\text{lag}})$. Consequently, the anomalous diffusion coefficient $\alpha$ can be directly extracted from the slope of this curve.

### 2.6. Used numbers

All simulations were carried out under periodic boundary conditions to reflect an infinite reservoir. The side length of the simulated square was set to 750 nm, which ensured that all observed results were insensitive to changes in the size of the simulated area. To emulate the size of large transmembrane proteins including a ring of annular lipids [16, 23, 24] or hypothesized lipid rafts [25], nanofeatures were simulated with a radius $R = \text{2 nm} \text{ to } \text{15 nm}$. The tracer diffusion constant in absence of nanofeatures was set to $D_{\text{out}} = 0.4 \, \mu\text{m}^2 \text{ s}^{-1}$, a typical value for lipid diffusion in the plasma membrane of a living cell [26].

### 3. Results

In this manuscript, we used a Monte Carlo simulation approach to study the diffusional properties of membrane molecules moving in distinct membrane environments. This reflects a situation that can be experimentally realized in a micropatterning experiment. In such an experiment, a circular ON-region is defined by specific enrichment and immobilization of nanofeatures, whereas the surrounding OFF-region is left unchanged (figure 1(A)). In a previous publication, we have analyzed the effect of immobilized obstacles on the diffusional properties of a tracer molecule focusing on steric...
hinderance and obstacle-tracer interactions [20]. Here, we went beyond this aspect and simulated nanofeatures, which are permeable to the tracer and slow down its diffusional motion. In the absence of any nanofeatures, the tracers were simulated with a diffusion constant $D_{\text{out}}$. Nanofeatures were assumed either as circular segments with a reduced diffusion constant $D_{\text{in}}$ (type 1) or inert obstacles with a surrounding ring characterized by $D_{\text{in}}$ (type 2). If not noted otherwise, nanofeatures were distributed uniformly and allowed to overlap. Throughout this paper, we refer to the density of nanofeatures either in terms of number density $\rho$ or in terms of area fraction $C$ (see Methods).

In principle, tracer diffusion was characterized by two figures of merit:

(i) The surface density ratio $\rho_{\text{ON}}/\rho_{\text{OFF}}$ measures whether tracers become enriched or depleted in the ON-region due to the presence of nanofeatures. For this, we determined the number of tracers in the ON-region ($N_{\text{ON}}$) and the OFF-region ($N_{\text{OFF}}$) and divided them by the total ON- and OFF-area ($A_{\text{ON}}$ and $A_{\text{OFF}}$), yielding tracer surface densities $\rho_{\text{ON}} = \frac{N_{\text{ON}}}{A_{\text{ON}}}$ and $\rho_{\text{OFF}} = \frac{N_{\text{OFF}}}{A_{\text{OFF}}}$. Note that tracers were simulated with equal partitioning into different membrane segments.

(ii) The mobility ratio $D_{\text{ON}}/D_{\text{OFF}}$ of tracers diffusing in the ON- and OFF-region reports on changes in tracer diffusion in dependence of nanofeature density and type. In the absence of nanofeatures, the diffusion constant $D_{\text{OFF}}$ is by definition equal to the diffusion constant $D_{\text{out}}$ outside of nanofeatures. We determined the diffusion constant $D_{\text{ON}}$ by analysis of the mean square displacement as a function of the time lag $t_{\text{lag}}$ according to $D = \frac{\text{msd}}{4 t_{\text{lag}}}$. 

Table 1. Summary of important variable names.

| Symbol | Definition |
|--------|------------|
| $\alpha$ | Anomalous diffusion exponent |
| $\beta$ | Time fraction tracers spend inside the nanofeature segments of low diffusivity $D_{\text{in}}$ |
| $C$ | Area fraction covered by nanofeatures |
| $C_{\text{core}}$ | Area fraction covered by impermeable nanofeature cores |
| $C_{\text{mem}}$ | Area fraction covered by membrane segments of low diffusivity |
| $d$ | Distance between a tracer and the closest nanofeature center |
| $D_{\text{in}}, D_{\text{out}}$ | Input diffusion constants inside and outside of nanofeatures, respectively |
| $D_{\text{ON}}, D_{\text{OFF}}$ | Resulting diffusion constants in ON- and OFF-regions, respectively |
| $D_0$ | Tracer mobility ratio $D_{\text{ON}}/D_{\text{OFF}}$ in the limit of short time lags ($t_{\text{lag}} \to 0$) |
| $D'_{\text{st}}$ | Tracer mobility ratio $D_{\text{ON}}/D_{\text{OFF}}$ in the limit of long time lags ($t_{\text{lag}} \to \infty$) |
| $N_C$ | Number of nanofeature clusters in the simulated two-dimensional space |
| $R$ | Nanofeature radius |
| $R_{\text{app}}$ | Radius of impermeable nanofeature core (for type 2 nanofeatures) |
| $\bar{r}(t)$ | 2D tracer position at time $t$ |
| $r^2(t_{\text{lag}})$ | Tracer square displacement |
| $\rho$ | Nanofeature number density |
| $\rho_{\text{ON}}$, $\rho_{\text{OFF}}$ | Surface density of tracers in ON- and OFF-regions, respectively |
| $\sigma$ | Standard deviation of Gaussian shaped nanofeature clusters |
| $t_{\text{delay}}$ | Time interval between two consecutive tracer observations |
| $t_{\text{lag}}$ | Multiples of $t_{\text{delay}}$ |
| $\tau_{\text{in}}, \tau_{\text{out}}$ | Mean time tracers spend inside or outside nanofeature segments of low diffusivity, respectively |

3.1. Domains of reduced mobility

First, we were interested in the effects of immobilized type 1 nanofeatures on tracer mobility. A possible realization of this scenario would be immobilized lipid rafts in a micropatterning experiment. We simulated circular nanofeatures with a radius $R = 8 \text{ nm}$ and a reduced diffusion constant $D_{\text{in}} < D_{\text{out}}$. We performed simulations for increasing densities of nanofeatures and analyzed both the tracer surface density ratios $\rho_{\text{ON}}/\rho_{\text{OFF}}$ and mobility ratios $D_{\text{ON}}/D_{\text{OFF}}$ as a function of the nanofeature density $C$. We did not observe changes in the surface density ratio with increasing nanofeature density for any tested ratio of diffusion constants $D_{\text{in}}/D_{\text{out}}$ (figure 3(A)). Since pure tracer retardation is insufficient for enrichment [27], this serves as validation of the ballistic transport model.

Plotting the mobility ratio $D_{\text{ON}}/D_{\text{OFF}}$ as a function of $t_{\text{lag}}$, revealed anomalous subdiffusion according to $D_{\text{ON}}/D_{\text{OFF}} \propto t_{\text{lag}}^{-\alpha}$, with $\alpha < 1$, over three orders of magnitude (figure 3(B)).

In the limit of very short and very long time lags, we observed apparent Brownian motion with $\alpha = 1$. Fitting the curves for different nanofeature densities $C$ with equation (3) yielded the plateau values of $D_{\text{ON}}/D_{\text{OFF}}$ and the degree of anomalous diffusion $\alpha$. We observed substantial deviation from Brownian motion with $\alpha$ down to 0.92 for large mobility differences between inside and outside of the nanofeatures (supplementary figures 1(A) and (B)).

In the limit of short lag times, tracers are limited to exploring their immediate surroundings typically without crossing nanofeature borders, yielding a plateau of pure Brownian motion with a mobility ratio $D_0 = \lim_{t_{\text{lag}} \to 0} D_{\text{ON}}/D_{\text{OFF}}$. In this limit, the dependence of $D_0$ on the nanofeature density followed a weighted average of the area fractions with diffusion...
Figure 3. Effects of different type 1 nanofeature densities. Simulations were carried out for diffusivity ratios $D_{in}/D_{out} = 1/3$ (Δ, dashed-dotted line in C and D), $D_{in}/D_{out} = 1/5$ (+, dashed line in C and D) and $D_{in}/D_{out} = 1/10$ (●, full line in C and D). Colors correspond to the simulated nanofeature density C. (A) Tracer surface density ratio as a function of nanofeature density C. Dashed line corresponds to equal tracer densities with $\rho_{ON}/\rho_{OFF} = 1$. (B) Tracer mobility ratio as a function of $t_{\text{lag}}$ for nanofeatures with $D_{in}/D_{out} = 1/10$. Dashed line corresponds to equal mobility with $D_{ON}/D_{OFF} = 1$. Full lines denote sigmoidal fits to the data using equation (3) (C, D) Mobility ratios are plotted as a function of nanofeature density C. Plateau values for $t_{\text{lag}} \to 0$ (C) and $t_{\text{lag}} \to \infty$ (D) were extracted from sigmoidal fits (equation (4) and (5)). Lines denote the empirical models for $D'_0$ (equation (6)) and $D'_\infty$ (equation (7)).

The difference between $D'_\infty$ and $D'_0$ can be interpreted as a measure of the spatial heterogeneity of the diffusion constant [28, 29]. Varying the size of simulated membrane domains merely shifted the regions of anomalous subdiffusion in time without notable effect on $D'_0$ and $D'_\infty$ (supplementary figure 2). To facilitate comparison with experimental data we also plotted the mobility ratio $D_{ON}/D_{OFF}$ versus the number density $\rho$ (supplementary figures 3(A)–(D) (available online at stacks.iop.org/JPD/53/435401/mmedia)).

In a real-life experiment, micropatterned nanofeatures are often not homogeneously distributed across the areas of enrichment. To elucidate the effect of such imperfect patterns we simulated clustered distributions of nanofeatures (figure 4(A)). Especially at low densities, the influence of clustering on the mobility ratio $D'_\infty$ was negligible (figure 4(B)). Note that for clustered nanofeatures at high densities $\rho$, the effective area fraction covered by nanofeatures differed significantly from $C = 1 - \exp\left(-\rho \pi R^2\right)$ due to increased overlapping of nanofeatures. Consequently, plotting the mobility ratio $D_{ON}/D_{OFF}$ as a function of the number density $\rho$ yielded
Figure 4. Effects of type 1 nanofeature clustering. (A) Exemplary distribution of clustered nanofeatures. Scale bare corresponds to 200 nm. (B) Long range mobility ratio $D'_\infty$ as a function of nanofeature density $C$ shown for mild ($N_C = 5$ and $\sigma = 200$ nm; +) and pronounced clustering ($N_C = 10$ and $\sigma = 100$ nm; ○). Colors correspond to the simulated nanofeature density. Full line denotes the empirical model for $D'_\infty$ (equation (7)). Arrow indicates the scenario shown in A.

Figure 5. Effects of spontaneously forming type 1 nanofeatures. Simulations were carried out for the diffusivity ratio $D_{in}/D_{out} = 1/10$. Colors correspond to the simulated time fractions $\beta = \tau_{in}/(\tau_{in} + \tau_{out})$. (A) Tracer mobility ratio is plotted as a function of time lag. Dashed line corresponds to equal mobility with $D_{ON}/D_{OFF} = 1$. (B) Average mobility ratio is plotted as a function of time fraction $\beta$. Full line denotes the empirical model for $D'_0$ (equation (6)).

It turned out to be interesting to briefly explore the situation of spontaneously forming nanofeatures, which would correspond to dynamically forming lipid rafts. Again, we assumed these rafts to form exclusively in the ON-regions of the micropatterning experiment. To this end, we simulated heterogeneity in diffusion based on a probabilistic algorithm. Tracers were allowed to randomly switch between different mobilities $D_{in}$ and $D_{out}$ with characteristic transition times $\tau_{in}$ and $\tau_{out}$, respectively. The fraction of tracers associated with a nanofeature is given by the time fraction $\beta = \tau_{in}/(\tau_{in} + \tau_{out})$ and directly corresponds to the area fraction $C$ in the model of immobilized nanofeatures. Surprisingly, we did not observe any anomalous subdiffusion in this scenario, and the mobility ratio $D_{ON}/D_{OFF}$ stayed at $D'_0$ for all simulated time lags (figure 5).

3.2. Inert obstacles with a surrounding ring of reduced mobility

On the nanoscale, transmembrane proteins associate with an annular shell of lipids, reducing the diffusivity around the protein up to several nanometers from the protein surface. To study the effects of immobilization and enrichment of such proteins in micropatterning experiments, we simulated ON-regions with different densities of inert obstacles with radius $R_{core}$ featuring a surrounding ring of radius $R$ with reduced diffusion constant $D_{in} < D_{out}$ (type 2 nanofeatures) (figure 1(B)). We tested several combinations of radii $R_{core}$ as well as mobility ratios $D_{in}/D_{out}$.

As expected, tracers were depleted from the ON-region with increasing nanofeature density $C$ (figure 6(A)). Tracer surface density ratios followed the relationship...
Figure 6. Effects of different type 2 nanofeature densities. Simulations were run for different combinations of ratios for radii $R_{core}/R$ and diffusivity ratios $D_{in}/D_{out}$. $igcirc$ and full lines correspond to $R_{core}/R = 5/8$ and $D_{in}/D_{out} = 1/10$. $+$ and dashed lines correspond to $R_{core}/R = 5/8$ and $D_{in}/D_{out} = 1/10$. $\Delta$ and dashed-dotted lines correspond to $R_{core}/R = 5/8$ and $D_{in}/D_{out} = 1/3$. Black filled circles denote the size exclusion model as described by equation (8). (A) Tracer surface density ratio as a function of type 2 nanofeature density $C$. (B) Tracer mobility ratio as a function of time lag for type 2 nanofeatures with $R_{core}/R = 5/8$ and $D_{in}/D_{out} = 1/10$. Dashed line corresponds to equal mobility with $D_{ON}/D_{OFF} = 1$. Full lines denote sigmoidal fits to the data using equation (3) (C, D) Mobility ratios are plotted as a function of type 2 nanofeature density $C$. Plateau values for $t_{\text{lag}} \to 0$ (C) and $t_{\text{lag}} \to \infty$ (D) were extracted from sigmoidal fits (equation (4) and (5)). Lines denote the empirical models for $D_0$ (equation (6)) and $D_\infty$ (equation (7)).

$$\rho_{ON}/\rho_{OFF} = 1 - C_{core}$$  \hspace{1cm} (8)

with $C_{core} = 1 - (1 - C)R_{core}^2/R^2$ denoting the area fraction covered by the solid obstacle cores (see Methods).

Plotting the mobility ratio $D_{ON}/D_{OFF}$ as a function of $t_{\text{lag}}$ showed anomalous subdiffusion with $\alpha < 1$. Particularly at high nanofeature densities, the degree of anomalous subdiffusion was higher than in the case of nanofeatures without a solid obstacle core (supplementary figures 1(C) and (D)). Considering the inaccessible area fraction $C_{core}$, the mobility ratio at short time lags followed the weighted average

$$D_0 = 1 - \left( 1 - \frac{D_{in}}{D_{out}} \right) \cdot C_{in}$$  \hspace{1cm} (9)

with $C_{in}' = C_{in}/C_{core}'$ denoting the fraction of accessible area covered by the viscous rings (see Methods) (figure 6(C)). With increasing density, tracer movements over long distances become increasingly impeded by impermeable obstacle cores until $C_{core}$ reached the percolation threshold $C_P = 0.676$ [30] and long range paths were completely blocked (figure 6(D)). The mobility ratio at long time lags could be described by a combination of the models for inert obstacles and nanofeatures with reduced diffusivity according to the empirical equation

$$D_\infty' = \left( 1 - \frac{C_{core}}{C_P} \right) \cdot \left( D_0' + \left( 1 - \frac{D_{in}}{D_{out}} \right)^2 \cdot \left( C_{in}'^2 - C_{in}' \right) \right)$$  \hspace{1cm} (10)

We also show the plateau values of the mobility ratio $D_{ON}/D_{OFF}$ in dependence of the number density $\rho$ to facilitate comparison with experimental results in supplementary figure 4.

3.3. Determining feature sizes with a model of inert obstacles

It turns out to be experimentally difficult to achieve nanofeature densities above $C \approx 0.4$ [17, 18]. Especially at low nanofeature densities, however, the ratio $D_{in}/D_{out}$ and
the nanofeature sizes $R$ and $R_{\text{core}}$ have similar effects on the curves, making it difficult to discriminate different nanofeature types based on recording only $D_{\text{eff}}$. Without additional knowledge about the nanofeature morphology, a practical approach may be to plot $D_{\text{eff}}$ as a function of $\rho$ and fit the data with a model of inert circular obstacles, yielding the apparent obstacle size $R_{\text{app}}$ as a fit result. In the following, we attempted to analyze data for type 1 or type 2 nanofeatures by fitting with a model for inert obstacles.

In figure 7(A) we show the results for type 1 nanofeatures (here circles with radius $R = 8$ nm and diffusion constant $D_{\text{in}} = 0.1 \cdot D_{\text{out}}$; full line), compared to a model for inert obstacles (radius $R = 7.94$ nm; dashed line). Expectedly, considering typical experimental noise the two curves would be experimentally indistinguishable. In this case, the relative error $\frac{D_{\text{app}} - D}{D}$ = -0.008 would be rather small. However, as shown in figure 7(B), the relative error may amount to much larger values for more faint differences in the diffusivity inside versus outside nanofeatures (black line). We also included type 2 nanofeatures (i.e. nanofeatures with an impermeable core): in this case, the inert obstacle model senses the effect of the core appropriately, yielding overall smaller deviations from the total size of the nanofeature.

4. Discussion

We provided here a first detailed theoretical evaluation of a hypothetical life cell experiment, in which viscous nanofeatures are immobilized at various surface densities in micrometer-sized patterns within the plasma membrane. We quantified the effect of different nanofeature morphology and size on tracer mobility and surface density, yielding the following key results:

(i) As expected, the tracer density ratio $\rho_{\text{ON}}/\rho_{\text{OFF}}$ is not affected by permeable nanofeatures (type 1), irrespective of the nanofeature diffusivity $D_{\text{in}}$. This is in line with the common definition of a partition coefficient, which is independent of probe mobility [27]. In other words, in the absence of size exclusion or chemical attraction or repulsion, probe molecules would distribute homogenously over phase-separated membranes.

(ii) Intuitively, one may have expected $D_{\text{ON}}/D_{\text{OFF}}$ to be a weighted average of the diffusivities inside and outside the nanofeatures. While this holds true for the short-range mobility ratio $D_0$, we found an interesting non-linear dependence of long-range mobility ratio $D_{\infty}$ on the nanofeature density (figures 3(C) and (D)). A similar phenomenon was previously reported in case of spatial heterogeneities in the diffusion constant [28, 29]. The non-linear dependence of $D_{\infty}$ on nanofeature density $C$ is a consequence of anomalous subdiffusion in the presence of substantial heterogeneity in the diffusivity. This happens to be pronounced particularly at intermediate nanofeature densities (supplementary figure 1). Notably, this non-linear dependence vanished in case of spontaneously forming nanofeatures (figure 5), indicating that the stability of the spatial diffusivity map is a precondition for this phenomenon.

(iii) We finally considered the situation of an experimentalist, who has recorded the $\rho$-dependence of $D_{\infty}$ without further knowledge about the underlying nanofeature morphology. According to Ockham’s razor, the experimentalist may opt for fitting the simplest model, which would be the presence of immobilized impermeable obstacles, yielding the apparent obstacle radius as result. We found that under conditions of substantial diffusivity reduction inside the nanofeatures this value closely reflects the actual extension of the nanofeature (figure 7).

Our simulations are based on a few simplifying assumptions, which shall be discussed in the following.

(i) Consistently with our previous publication on the effects of inert immobile obstacles, we assumed point-like tracers throughout this manuscript. The experimental realization
could be a single fluorescently labelled lipid molecule, which has a typical in-plane radius of less than 5 Å. (ii) While theoretically interesting, the assumption of immobilized type 1 nanofeatures may be experimentally difficult to achieve. Our underlying goal was to study the effects of raft immobilization on the mobility of lipid tracers. However, for experimental reasons the immobilization of the putative raft requires the presence of raft-resident markers, which can be captured on micropatterns. In most realizations, such a marker corresponds to a membrane protein, which constitutes an impermeable obstacle within the nanofeature; we termed this scenario nanofeature type 2. Still, in the case of a GPI-anchored protein this obstacle core can be fairly small (two fatty acid chains amounting to approximately 5 Å radius) compared to the nanofeature radius, hence closely resembling the situation of a type 1 nanofeature. (iii) We assumed here discrete transitions between $D_{in}$ and $D_{out}$ at the contour of the nanofeatures. This assumption simplifies putative fluctuations of the contours in case of lipid phase separation, or gradients in diffusivity in case of boundary lipids. While potentially having minor quantitative effects, we do not expect qualitative changes on the presented results. (iv) In this paper, we considered flat membranes. This appears justified at the bottom membrane of a cell anchored to the surface of a glass coverslip. Still, slight membrane undulations could be present, which would lead to a retardation of the apparent diffusion constant when measured via two-dimensional single particle tracking [31]. However, due to the high density of anchored nanofeatures in typical experimental settings, we do not expect substantial effects due to three-dimensional membrane topology. Ultimately, the problem could be approached by measuring the three-dimensional diffusion, e.g. via supercritical angle fluorescence microscopy [32].

In conclusion, diffusion analysis in conjunction with a micropatterning experiment provides valuable information on the size of immobilized nanofeatures. Recording and including additional information such as tracer density ratios, and tracer diffusivities inside versus outside of the nanofeatures would further help to determine nanofeature dimensions more precisely.

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