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Review

Life in crowded conditions

Molecular crowding and beyond

Stefan Klumpp[a], William Bode, and Palka Puri

Institute for Nonlinear Dynamics, University of Göttingen, Friedrich-Hund-Platz 1, 37077 Göttingen, Germany

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Abstract. Molecular crowding is ubiquitous in cells, which are rather densely packed with macromolecules. The effects of such crowded conditions on biophysical processes can be complex and puzzling. Here, we review these effects in a step-by-step manner. We start with excluded volume effects on elementary physical processes: diffusion, binding, reactions, and polymer compaction. We then discuss the binding of a transcription factor to a binding site on DNA as an example of a more complex process and consider effects of attractive interactions and active processes. We also give an outlook to larger-scale crowded systems such as suspensions of cells, biofilms, and tissues, which can be described using similar approaches as molecular crowded systems.

1 Introduction

The cytoplasm of living cells is a crowded environment, densely packed with macromolecules at volume fractions of up to 40% [1,2]. This so-called macromolecular crowding is a thus a ubiquitous ingredient in all processes of life and has the potential to affect their molecular mechanisms. Despite its ubiquity, molecular crowding is often underappreciated [2]. The standard conditions for the investigation of biomolecular processes are dilute solutions, quite different from the conditions in cells. Obviously, the use of dilute solutions can be justified by a reductionist agenda with the desire to study and understand the individual components and minimal functional systems before addressing the complexity of a whole cell. Nevertheless, eventually the impact of the high density needs to be addressed and this is again done by studying biomolecular processes in simplified systems containing crowding agents [3], typically a high concentration of a single type of inert molecules. However, one needs to keep in mind that even if these systems exhibit the high density of the cytoplasm, they are quite different from cytoplasm in many other aspects. In particular, cytoplasm is very heterogeneous with large numbers of different types of molecules of very different sizes. Moreover, the cytoplasm is heterogeneous spatially, with different compositions in different areas, as well as dynamically, with a wide range of different mobilities of the different types of molecules.

[a] e-mail: stefan.klumpp@phys.uni-goettingen.de

The spatial heterogeneity of the cytoplasm and the wide variety of molecular species is documented by catalogues of its composition, see e.g., reference [4], and by high-resolution imaging through cryo-electron tomography [5].[1] The dynamic heterogeneity is to some extent seen by fluorescence microscopy, tracking fluorescently labeled molecules in the cell [7,8]. It is also observed in large-scale simulations of molecularly resolved cytoplasm [9,10]. The possibility of large-scale simulations of crowded systems has certainly been one catalyst for the increased interest in crowding in recent years. In addition, studying crowding in cells became possible due to advances in fluorescent probe techniques which have led to the use of 'crowding sensors' to probe the degree of crowding in cells [11,12]. In principle, all processes that are affected by crowding can be used as such probes, but typically crowding has been probed either via the diffusion of tracer particles that are tagged by fluorescent molecules [8,13] or via the compaction of polymers as measured by Förster resonance energy transfer (FRET)-probes at the two ends of the polymer [11,12].

Crowders, the molecules in the background of a process of interest, may interact with the molecules of interest, e.g. the reaction partners of a chemical reaction, in many different ways, through attractive and repulsive interactions, which can make the effects of crowding very complex. One unavoidable component of the interaction is excluded volume, the short-ranged repulsive interaction that prevents that two molecules occupy the same volume. If the molecules are extended rather than point particles, the volume from which they exclude each other are larger than their own size as illustrated in Figure 1A. However, even the effects of excluded volume can be surprisingly complex, in particular, if excluded volume affects a process in multiple ways.

In this tutorial review, we will therefore build up complexity step by step and first discuss in Section 2 how several elementary processes are affected by crowders (binding, diffusion, enzymatic reactions, and polymer compaction). We will then discuss more complex processes in Section 3 and have a look beyond excluded volume in Sections 4 and 5, discussing the effects of attractive interactions and of active processes, respectively. Section 6 adds some remarks on crowding beyond *molecular* crowding, before we conclude with some general remarks. Aspects of crowding that we will not discuss here, in particular, specific biochemical aspects and crowding effects on protein folding, are reviewed in several other review articles such as references [14–17].

2 Basic effects of excluded volume

We start with the simplest case, in which crowders interact with the particles of interest only through excluded volume. While for many crowded systems, it is not clear whether this is indeed the only interaction, excluded volume is a type of interaction that cannot be avoided and is always present. Excluded volume affects binding equilibria, diffusion, and the compaction of polymers.

2.1 Binding

Generically, binding between any two binding partners is enhanced by the presence of crowders [2]. This can be understood qualitatively by considering the system as a two-state system, bound and unbound. The entropy of the unbound state is reduced by crowders as fewer microstates of the unbound particles are possible, thus shifting

[1] The crowded nature and the spatial heterogeneity of cytoplasm are also nicely illustrated by the realistic drawings of Goodsell [6].

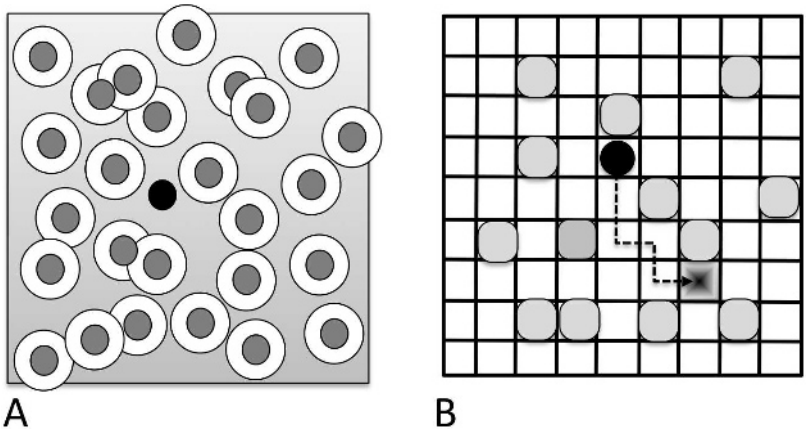

Fig. 1. Excluded volume: (A) Every crowder particle (grey) excludes the black particles from the spherical volume indicated in white. If these excluded volumes overlap, the total accessible volume increases and thereby the entropy of the black particle. This is also the source of depletion forces, attractive interactions between the larger particles. (B) Lattice model for crowding: particles occupy one lattice site each and exclude other particles from it. The black particle represents a ligand binding to a receptor (indicated by the site with a cross).

the equilibrium towards the bound state. To quantify this effect, one can consider a lattice model in which one site corresponds to one binding partner, say the binding pocked of a receptor [18,19], see Figure 1B. Other particles, N ligands binding to the receptor and M crowders, which simply occupy space, are then distributed on the lattice (V sites for unbound particles).[2] The relative weights of the bound and unbound state are given by the number of possible configurations of the unbound particles and a Boltzmann factor with the binding energy ϵ, i.e.

$$\begin{aligned} w_{\mathrm{b}} &= \binom{V}{N-1+M}\binom{N-1+M}{N-1}e^{-\epsilon/kT} \\ w_{\mathrm{ub}} &= \binom{V}{N+M}\binom{N+M}{N}. \end{aligned} \tag{1}$$

From this one can obtain the probability that the receptor is occupied as

$$P_{\mathrm{b}} = \frac{w_{\mathrm{b}}}{w_{\mathrm{b}} + w_{\mathrm{ub}}} \approx \frac{c}{c + e^{\epsilon/kT}(1-\phi)} \tag{2}$$

with the volume fraction of ligands $c = N/V$ and the volume fraction of crowders $\phi = M/V$. The approximation used here assumes that the system is dilute, but denser systems give similar results. Thus, binding equilibria are shifted towards lower concentrations of the ligands (Fig. 2) as indicated by the effective (crowding dependent) dissociation constant $K_d = e^{\epsilon/kT}(1-\phi)$.

It is also instructive to consider the kinetics of this process [19]: if particles hop from one lattice site to the neighboring one and become bound once they reach the receptor site, the binding rate is limited by diffusion. Since diffusion is slowed down by

[2] We note that all the models considered here assume that the solvent is an aqueous solution with properties that do not change as the volume fraction of crowders is modulated. For a study of the effect of crowders on solvent properties, see reference [20].

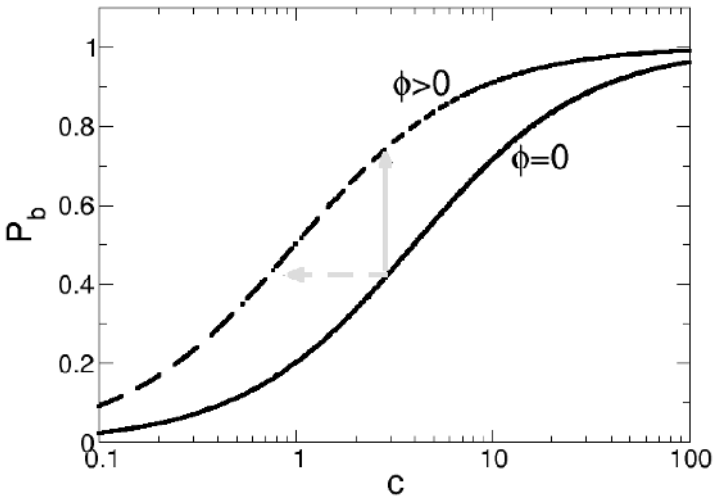

Fig. 2. Probability P_b that a binding site is occupied as a function of the concentration c of the ligands binding to that site. The presence of crowders ($\phi > 0$) shift the binding curve towards smaller concentrations c. Thus, the same occupancy of the binding site is achieved with a lower concentration or a higher occupancy at the same concentration, as indicated by the dashed and solid grey arrows, respectively.

crowding (in the lattice model, hops to occupied sites are simply rejected), one would expect that the binding rate is reduced. This is however not the case or only in parts. Analysis of the times a ligand is bound or no ligand is bound to the receptor shows that the average time to unbinding increases (because some unbinding attempts are prevented by the presence of crowders), but the average time to binding shows a convolution of two effects: binding in the presence of crowders is characterized by two time scales, which are affected by an increase in the crowder volume fraction in opposite ways. Slow binding of ligands that have to diffuse to the receptor from far away is indeed reduced as expected. However, rapid (re-)binding of a ligand near the receptor is enhanced by crowders, which now prevent that the ligand diffuses away and thereby provide more time for binding to happen. In coarse-grained simulations, one can therefore define an extended bound state that includes configurations where the ligand stays close to the receptor without diffusing away to obtain an effective two- or three-state model [21].

The shift in binding equilibrium is more pronounced and K_d decreases more rapidly with ϕ if the crowders are smaller than the ligands and the receptor. This size effect is due to depletion forces (Fig. 1), effective attractive interactions between larger particles (such as the ligand and the receptor) in the presence of small crowders.

A different perspective on the same process, more common in the biochemical literature, is obtained by considering the chemical potential of particles in a dense solution. If the solution was dilute, the chemical potential of a molecular species i could be written as $\mu_i^{(\mathrm{ideal})} = \mu_i^{(0)} + kT \ln c_i$, where c_i is the corresponding concentration. If the solution is not dilute, interactions between the particles (including excluded volume) need to be taken into account and add an interaction term to the free energy, this also results in an additional term in the chemical potential and leads to $\mu_i = \mu_i^{(\mathrm{ideal})} + kT \ln \gamma_i = \mu_i^{(0)} + kT \ln(c_i \gamma_i)$ [1,2]. The combination $c_i \gamma_i$ defines an effective concentration and is called 'thermodynamic activity' of species i, γ_i itself is called activity coefficient. As a consequence, the equilibrium constants of chemical reactions are modified by crowding by multiplying them with

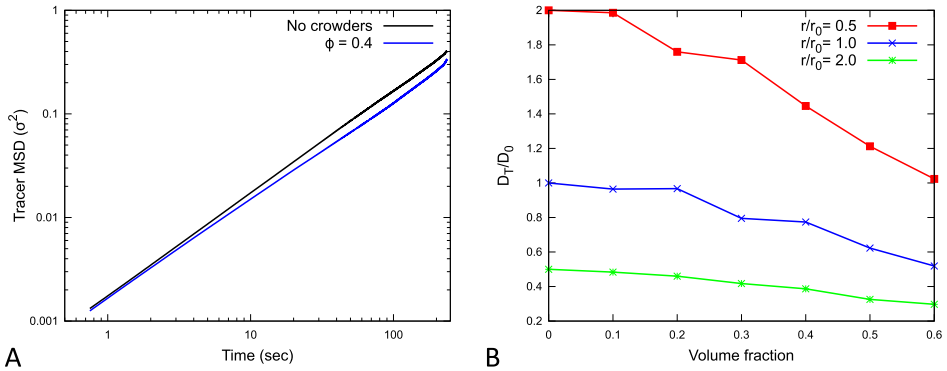

Fig. 3. Tracer diffusion in the presence of crowders. (A) Mean square displacement of a tracer particle as function of time in the absence and presence of crowders (with volume fractions $\phi = 0$ and $\phi = 0.4$, respectively). (B) Diffusion coefficient as function of the volume fraction of crowders, normalized to the diffusion coefficient D_0 of a single particle of size r_0. The crowders have that size in all three cases plotted. For the blue curve the tracer has the same size ($r = r_0$), for the other two curves, the tracer is smaller ($r = 0.5r_0$) or larger ($r = 2r_0$), respectively. All data are from Brownian dynamics simulations of almost hard spherical particles in two dimensions. The particles interact through the repulsive part of a Lennard–Jones potential and are subject to thermal noise, which results in the single particle diffusion coefficient D_0. The simulations were run in a two-dimensional system of size $24r_0 \times 24r_0$, the used volume fractions thus correspond to 1–110 particles. The diffusion of a single tracer particle was tracked to obtain the diffusion coefficient by sliding window average over a long trajectory. Periodic boundary conditions were used for the positions of the crowders and to calculate the interaction of the tracer with the crowders, but the position of the tracer particle was tracked without the use of the periodic boundary conditions, so displacements exceeding the system size are possible.

the corresponding combination of activity coefficients. For example, the formation of a complex, $A + B \rightleftharpoons C$ in equilibrium is characterized by $K_d = K_d^{(\mathrm{ideal})} \frac{\gamma_C}{\gamma_A \gamma_B}$. Assuming that the $\gamma_{A,B,C} \approx \gamma$ for typical proteins, Morelli et al. have estimated how much dissociation constants in cells are decreased compared to in vitro experiments and found $\gamma \simeq 10 - 100$, indicating that binding can be strengthened considerably in cells [22].

2.2 Diffusion

A second elementary physical process affected by crowding is diffusion. Simulations show that on long time scales, the mean square displacement of particles in the presence of crowders exhibits a linear dependence on time, the slope of which defines an effective diffusion coefficient. This diffusion coefficient decreases with increasing volume fraction of crowders, as shown in Figure 3. As in the case of binding discussed above, the effect is more pronounced for larger particles in the presence of smaller crowders.

In the lattice model with particles occupying exactly one lattice site, the reduction of diffusion is particularly simple, as volume exclusion simply prohibits the hopping of particles to occupied sites, thus the diffusion coefficient is reduced in a linear manner, $D(\phi) = D_0(1 - \phi)$. In general, the reduction of diffusion can be expressed via the free energy required to free the target volume of crowders, ΔG^*, as $D = D_0 e^{-\Delta G*/kT}$. Explicit expressions for $\Delta G*$ have been given for spherical particles,

$\Delta G^*/kT = -\ln(1-\phi) + f(Q)$, where $f(Q)$ is a series expansion in $Q = \phi/(1-\phi)$ [15,23]. An empirical expression for the reduction of diffusion is given by $D = D_0\gamma^{-\kappa} \approx D_0(1-\phi)^{\kappa}$ with $\kappa \simeq 0.36$ estimated from diffusion of proteins in dense protein solutions [22] and $\gamma = 1/(1-\phi)$ and $\kappa \simeq 0.5$ in the lattice model [19].

Diffusion measurements in cells indeed show that diffusion is typically slower in the cytoplasm than in dilute solutions, with a strong size-dependence [24]. For instance, diffusion of GFP in bacterial cytoplasm is about one order of magnitude lower than in an aqueous solution [25]. Upon osmotic shock, which strongly increases crowding, diffusion is slowed down further, but recovers partially as the cells adapt [13]. The reduction of diffusion is seen to be strongly size dependent, both in experiments [7] and in computer simulations [10].

As a consequence of the slowing of diffusion, chemical reactions that are diffusion-limited will also have a reduced rate. An instructive alternative perspective on this is obtained by considering the kinetics of binding and of the reaction within the lattice model described above [19]. If the ligand is thought of as the substrate of an enzymatic reaction that is diffusion limited, the reaction takes place as soon as the ligand reaches the enzyme. This means that no repeated cycles of binding and unbinding take place before the reaction occurs. Rather diffusive binding of the ligand/substrate to the enzyme is reverted by the reaction itself. After the reaction, however, the ligand/substrate has been converted into a reaction product, so that no rapid rebinding of the substrate is possible. This means that among the two time scales of binding as discussed before, the rapid one is not present in this case, and only the slow one remains, which however becomes even slower as crowding is increased. In the lattice model, such a reaction can be implemented by merging the reaction itself, unbinding of the product and the necessary steps to keep the concentrations of substrate and product constant (removal of product plus regeneration of substrate) into a single event: in this single event a substrate bound to the enzyme unbinds to a random site on the lattice unbinding (rather than a neighboring site as for simple unbinding) [19].

The diffusion limit may apply to some biochemical reactions involving large molecular machines, such as binding of elongation factors (ternary complexes) to ribosomes and of RNA polymerases to highly active promoters. Binding of elongation factors to ribosomes (delivering the tRNAs with the amino acids to be incorporated into the protein that is synthesized) has been estimated to be close to the diffusion limit [26], but measurements of the translation speed [27] found values larger than expected based on those estimates. This means that the cell can likely avoid the diffusion limitation, possibly by a mechanism such as storing elongation factors and tRNA on the ribosome, for which there is indirect evidence from bioinformatics [28] and recent direct evidence from fluorescence microscopy [29].

In the situations we have discussed so far, diffusion in the crowded environment has a reduced diffusion coefficient, but is 'normal' in the sense that the mean square displacement of the tracer particle increases linearly with time in the long-time limit. In many crowded situations, tracers may also exhibit anomalous diffusion, i.e. a mean square displacement $\Delta x^2 \sim t^{\alpha}$, where $\alpha \neq 1$ [30], typically $\alpha < 1$ (subdiffusion) for crowded environments. As a transient process, subdiffusion is generic and should always be expected when diffusion is slowed by obstacles such as crowders, on intermediate time scales (on which the exponent α first decreases and then increases again). However, in a number of models [30], subdiffusion persists in the long-time limit with a constant exponent α, which would be the case of true anomalous diffusion. Such behavior has been observed in cells, see, e.g., references [31–33], and understanding the source of such anomalous diffusion is an active field of research. Some insights into the mechanisms of anomalous diffusion can be obtained by comparing ensemble averages of the mean square displacements of many trajectories and

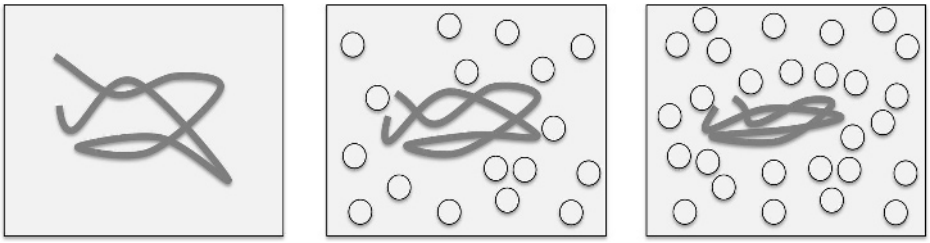

Fig. 4. Compaction of a polymer by increasing volume fraction of crowders.

moving-window time averages over individual trajectories, which are typically not equivalent in the case of anomalous diffusion [30].

2.3 Enzyme kinetics

Enzyme kinetics that consist of diffusion-limited binding and a subsequent reaction step interpolates between the two limiting cases that have already been mentioned, equilibrium binding and diffusion-limited binding followed by a rapid reaction. In the first limiting case, the reaction is sped up by crowding, as the formation of the enzyme-substrate complex is enhanced. In the other limiting case, the reaction is limited by diffusion, and is thus slowed down by crowders rather than sped up. In the general case, the first effect is dominant for small volume fractions of crowders and the second for large volume fractions. Thus, the reaction gets faster for small volume fractions up to a value where the maximal rate is reached. Beyond this volume fraction, the slowing of diffusion dominates and the reaction is slowed again.

2.4 Compaction of polymers and protein folding

Yet another process affected by crowding is the compaction of polymers. Crowded conditions typically lead to more compact configurations of the polymer as these reduce the volume from which the crowder particles are excluded (Fig. 4). For that reason, the compaction of polymers has been used as a 'crowding sensor' to be used to determine how crowded the conditions in the cytoplasm of cells actually are [11,12]. To that end, the two ends of a polymer were labeled with FRET probes to indicate when the ends are in close proximity. These sensors showed a systematic modulation by the volume fraction of inert crowders in dense suspensions in vitro, and indicated increased crowding in bacterial and mammalian cells when the osmotic pressure was increased [11,12]. In unstressed cells, the effect was however smaller than expected based on the in vitro results [11]. These experiments also showed that the degree of crowding is spatially heterogeneous in the cell.

Closely related to the compaction of polymers is the effect of crowding on the folding of proteins, which can be imagined as heteropolymers with additional attractive interactions between specific pairs of amino acids (native contacts) that give the protein is characteristic and functional three-dimensional shape. The general compaction helps the folding of the protein, so that typically the folded structure is stabilized by excluded volume interactions [15,34], although the details of the effect can be complex. An intermediate case between simple polymers and proteins with a well-defined three-dimensional structure is intrinsically disordered proteins, which only exhibit weak internal attractive interactions that are not sufficient to stabilize a well-defined native structure. These proteins are compacted by crowders just like polymers [35]

and for some of these proteins, this induces the formation of a well-defined structure that is not present in absence of crowders [36].

3 A composite process: search for binding sites by transcription factors

If a biochemical process consists of multiple sub-processes that are all affected by crowding in some way, the combined effects can become complex and hard to predict. For example, an enzymatic reaction might be inhibited by crowding, because it is limited by diffusion, but if that reaction depends on dimerization of the enzyme (or on formation of a larger enzyme complex), the rate is also increased by the enhanced binding that increases the concentration of enzyme in the correct dimeric configuration. The same is true if the stability of the enzyme's three-dimensional configuration is low and a positive effect of crowders stabilizing the enzyme's structure enhances the reaction.

As an example for a composite process, we consider binding of a transcription factor to its binding site on DNA. Transcription factors (as well as other DNA-binding proteins) bind to DNA in a sequence-specific manner at dedicated binding sites. In addition, however, they can also bind DNA in a non-specific manner, typically due to electrostatic interactions [37]. In the nonspecifically bound state, the transcription factors can slide on the DNA, i.e. perform one-dimensional diffusion. How the interplay of three-dimensional diffusion in the cytoplasm and one-dimensional diffusion on the DNA results in rapid binding to the functional (sequence-specific) binding site and how the search for that site is to be optimized has been studied extensively [37,38] and was shown to quantitatively describe diffusion of transcription factors in bacterial cells [39].

A basic picture of the effects of non-specific binding is obtained by considering binding to the target, the functional binding site as a diffusion-limited process that occurs with rate

$$k = \eta D a, \tag{3}$$

where D is the diffusion coefficient, a the size of the target, and η a geometric factor ($\eta = 4\pi$ in the original spherical geometry studied by Smoluchowski [40], and $\eta = 4$ for a cubic lattice model). Together with the concentration c of the transcription factor, the time τ for the search of the target is then obtained as the inverse of the rate $\tau = (\eta D a c)^{-1}$. The effects on non-specific binding can be included in this rate by recognizing two effects. On the one hand, binding to DNA far from the target site essentially pauses diffusion, thus an effective diffusion coefficient can be written as $D_{\mathrm{eff}} \approx D(1 - P_{\mathrm{ns}})$, where $P_{\mathrm{ns}} = [1 + k_{\mathrm{off}}/(\eta D a c_{\mathrm{DNA}})]^{-1}$ is the probability that the transcription factor is nonspecifically bound to DNA, assuming again a diffusion-limited binding rate with the concentration of DNA, c_{DNA}, and an unbinding rate k_{off}. On the other hand, binding close to the target has a beneficial effect, because a transcription factor that binds DNA close to the target may slide into the target. Thus, the diffusive search does not need to lead to the target directly, but rather to an increased target with effective size $a_{\mathrm{eff}} \approx \lambda$, where we have introduced the sliding length $\lambda = \sqrt{D_1/k_{\mathrm{off}}}$, the distance over which a non-specifically bound transcription factor slides on DNA (with the one-dimensional diffusion coefficient D_1, which is typically smaller than the cytoplasmic diffusion coefficient D) before unbinding. Thus, we obtain the rate

$$k = \eta D_{\mathrm{eff}} a_{\mathrm{eff}} = D a \eta \left[\frac{a}{\lambda} + \frac{a^2 \lambda D}{D_1} \eta c_{\mathrm{DNA}} \right]^{-1}. \tag{4}$$

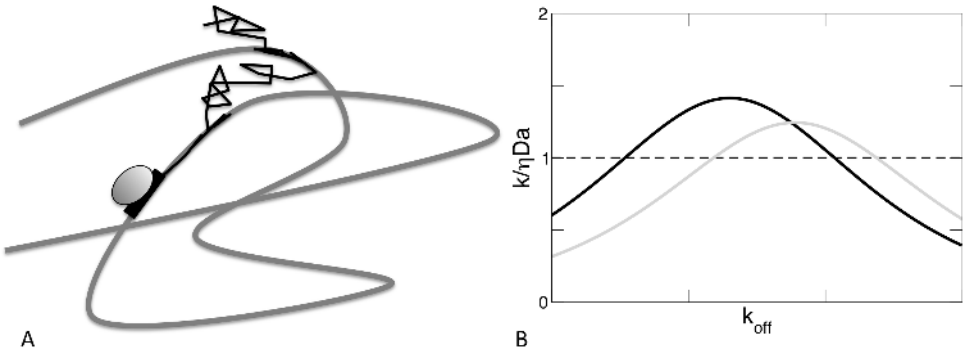

Fig. 5. Facilitated diffusion of a transcription factor searching a binding site on DNA. (A) Schematic depiction of the search process, (B) typical dependence of the binding rate on the strength of nonspecific binding to DNA as measured by the unbinding rate k_{off}, which can be varied with the salt concentration (schematic plot). The binding rate k is scaled with the diffusion-limited binding rate in the absence of crowding and nonspecific DNA binding ηDa, i.e. whenever the curves are above the dotted line, the search is faster due to the combination of 1D and 3D diffusion than by 3D diffusion alone ('facilitated diffusion'). Crowding shifts the optimal value of k_{off} (compare the grey curve to the black curve), thus it depends on the parameters whether crowding speeds up or slows down binding to the functional binding site.

The resulting rate is maximized by an optimal sliding length, where the search for the binding site is most rapid (Fig. 5). Experimentally, the sliding length is varied through the salt concentration, which modifies the unbinding rate k_{off}; this indeed shows the expected maximum of the binding rate [41]. The result given by equation 4, which was obtained based on estimating an effective diffusion coefficient and an effective target size, agrees with the results of more detailed calculations [38] up to numerical factors.

The effects of crowding on this combined diffusive process have been studied in a number of computational and analytical studies [42–47]. Crowding affects this process in multiple ways. It slows diffusion in the cytoplasm, but it also enhances non-specific binding to DNA. This in turn has two opposing effects: on the one hand, enhanced nonspecific binding slows down diffusion even further (via the decrease of D_{eff}); on the other hand, it increases the sliding length, as has been tested by simulations on and off lattice [44]. Thus together the two effects may either speed up the search for the target or slow it down, depending on the parameters of the system, as indicated schematically in Figure 5. If the system is optimized for search in dilute conditions, it can be expected to be slower under crowded conditions.

The effects of crowders bound to the DNA on the search for a binding site have also been considered, because many other proteins are bound to DNA, including other transcription factors and histones (or, in bacteria, nucleoid-binding proteins), and may act as obstacles for the sliding. In that case, the effect of the crowders is purely negative, as they restrict the sliding length by confining sliding to the area between two obstacles. However, the quantitative extent of this effect is strongly dependent on the dynamics of the obstacles, with the strongest effect due to static obstacles and smaller effects for obstacles that slide on DNA as well or even unbind transiently [45,46].

4 Beyond excluded volume: attractive interactions

So far, we have considered a scenario in which crowders interact with each other and with the crowding probes only through excluded volume. In the cell however,

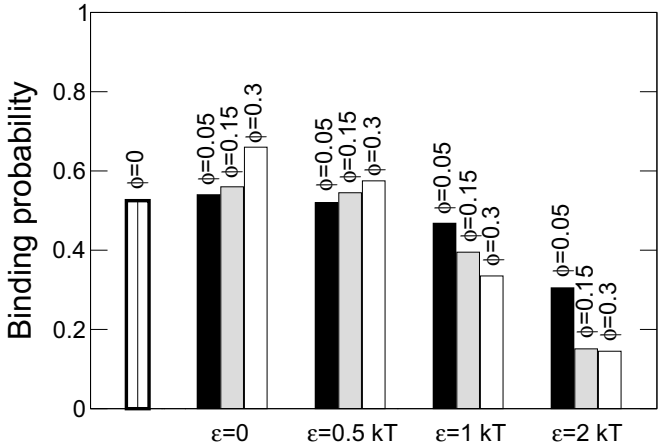

Fig. 6. Probability that a receptor is bound by a ligand in the presence of crowders with attractive interactions with the ligands: results from a lattice model with volume fraction ϕ of crowders, and attractive interaction energy between ligands and crowders on nearest neighbor sites ϵ. A weak attractive interaction can compensate the effect of excluded volume. In the lattice model, ligands and crowders perform random walks on a lattice and steps to a neighboring site are suppressed if the target site is occupied. Attractive interactions between a ligand and a crowder on neighboring sites (with energy ϵ) are included by performing a Metropolis criterion check before a move is performed. The results shown here are for a three-dimensional lattice of 512 sites that contains one immobile receptor site, a constant fraction (0.2) of mobile ligands and a fraction ϕ of sites occupied by mobile crowders.

additional interactions may play a role. In particular, weak non-specific attractive interactions are present between many different types of molecules. For example, many proteins bind nonspecifically to DNA due to electrostatic interactions [48]. Likewise, there are many weak protein–protein interactions, which have recently been proposed to constrain the evolution of protein–protein interaction networks [49].

In the lattice model, nonspecific attractive interactions can be included as a nearest neighbor interaction, e.g. between the ligands in a ligand-receptor binding reaction and the crowders. Whenever a particle is moved, the move is checked by a Metropolis criterion and accepted with probability one if the energy is decreased by the step and with probability $e^{-\Delta E/kT}$ if the energy would be increased by the step by an amount $\Delta E > 0$. The weak binding between the ligands and the crowders competes with binding of the ligands to the receptor. Surprisingly, very weak attraction between the ligands and the crowders is sufficient to compensate the effect of excluded volume, which enhances binding to the receptor.[3] Figure 6 shows the effect of excluded volume as well as weak attraction with energy ϵ between ligands and crowders on neighboring lattice sites. Pure excluded volume interaction ($\epsilon = 0$) enhances receptor-ligand binding, but already very weak attraction ($\epsilon = 0.5kT$) approximately compensates the excluded volume effect. For stronger interactions, the attraction dominates over excluded volume and the receptor-ligand binding is weakened.

Nonspecific interactions can also modify the stabilizing effect of crowding on protein folding. Indeed, crowded conditions have been observed to have stabilizing or destabilizing effects on different proteins, depending on the protein under consideration as well as the type of environment, for example, the type of crowders or

[3]That many weak interactions dominate over a single stronger interaction is reminiscent of the DNA-binding of RNA polymerase, which also seems to be dominated by weak binding at many nonspecific sites rather than stronger specific binding at a smaller number of promoters [50].

the cellular compartment. These effects are discussed in more detail in references [3,51,52].

Finally, we mention that some of the weak interactions that are called 'nonspecific' may actually have a function (similar to the nonspecific binding of transcription factors to DNA that we discussed above) rather than just being nonfunctional perturbations of the stronger functional interactions. One hypothesis with some recent experimental support is that weak interactions link enzymes with functions within one pathway into a complex, in which substrates and products can be channeled from one enzyme to the next as in an assembly line with the potential to speed up the reaction [53,54]. Support for such an idea is also provided by observations on overexpression toxicity: overexpression of some genes is toxic and related to weak interactions with essential enzymes, but this toxicity is absent if the homologous genes from other organisms are used [55]. These observations indicate that weak interactions are species-specific and have been selected by evolution. Moreover, weak interactions also seem to be key for the (liquid–liquid) phase separation that is increasingly recognized as a organization principle of the cytoplasm, where weakly sticking proteins and RNAs form dynamic droplets that act as membrane-less compartments within a cell [56,57]. This types of compartments have also been proposed as candidates protocells in the early evolution of life [58].

5 Crowding involving active processes

The cytoplasm is not a suspension in thermodynamic equilibrium. Rather, objects are moved actively by molecular motors and pumps [59]. These active processes also contribute to the effects of crowding, as a certain fraction of the crowders in the cell are active molecules. There are many different ways how activity can manifest itself, but the best studied one is self-propelled motion of particles. In the cell, this mostly affects molecular motors and larger particle pulled by molecular motors, in particular the motors of the cytoskeleton as well as motors moving on DNA or RNA such as RNA polymerases and ribosomes. When these motors collide with a molecule of interest, they will exert a force on it that in contrast to thermal collisions has a certain persistence, that is the force is exerted in the same direction for some time [60]. This scenario has not been studied much at the molecular level, but it was studied at the level of suspensions of cells, in particular swimming bacteria [61–63]. In this case, the active crowders typically increase the diffusion of passive tracer particles rather than decreasing it, as they decrease their passive diffusion, but at the same time induce an active contribution to the diffusion which can exceed the passive part by orders of magnitude. While these systems have originally been studied in the presence of hydrodynamic interactions rather than the simple excluded volume we discussed here, recent studies have shown that hydrodynamic interactions are not essential and that self-propulsion plus excluded volume alone leads to results that are qualitatively the same [64,65].

In addition to self-propulsion, active particles may also simply exhibit a diffusion coefficient that is different from the one expected based on the Stokes–Einstein relation, typically one that exceeds that expectation. Enhanced diffusion due to an active process has been reported for enzymes, with considerable debate about the mechanism [66–68]. The effects of such active crowders on passive tracer particles have only occasionally been addressed.

Clear evidence for a role of active processes (though not for a specific type of active processes) is provided by the observation that diffusion in bacterial cells is dependent on the cell's energy status: diffusion of large particles in the cytoplasm was shown to be strongly reduced upon depletion of energy [8]. The slow diffusion

also showed non-ergodic behavior, a common observation in cells, often in the context of subdiffusion [32]. This slowing of diffusion has been described as a transition from a fluid state of the cytoplasm to a 'glassy' or jammed state corresponding to an amorphous solid. Similar observations were made for diffusion in eukaryotic cells during cell division, where diffusion was found to be anisotropic and reduced only in the direction perpendicular to the axis of the mitotic spindle [33], again possibly due to active fluidization by molecular motors along the direction of the mitotic spindle axis.

6 Outlook: crowding on larger scales: cells, tissues, organisms

When discussing active processes, we have already mentioned that effects of collisions with active particles have mostly been discussed at larger scales, specifically for suspensions of bacteria, in which the diffusion of passive tracer particles is enhanced rather than obstructed by a high density of the active crowders [61–63]. Indeed, crowded conditions are quite common in cellular systems as well, where the proliferation of cells increases the density, for example in suspensions of cells, colonies, biofilms, or tissues. Two scenarios can be distinguished: crowded systems of hard particles such as bacterial cell and crowded systems of soft particles such as many eukaryotic cells. In the latter case, the density is of lesser importance, as cells typically fill the space in tissues, and the main control parameters are the cell–cell adhesion and the tension in the cells' cortex [69]. For hard particles such as bacteria, on the other hand, the density or volume fraction is the most important control parameter as in the molecular systems we have discussed so far.

Active processes are also of importance for cellular-scale crowded systems. One example is the self-propulsion that we have already discussed, both for suspensions and for tissues. Another example is proliferation itself, which moves cells by pushing them away and can increase the density up to a jammed state [70]. Likewise, cell division and cell death can be a source for the fluidization of tissues [71]. The pushing of cells by proliferation also leads to the complex patterns that nonspherical cells form in the early stages of biofilm growth [72,73].

7 Concluding remarks

Crowded conditions are ubiquitous in biological systems, from the molecular crowding in cells to high densities in colonies of cells and tissues. The effects of molecular crowding have been studied for a long time, but have not always received the recognition they deserve. This has changed over the years, in particular with respect to crowding in cells, as the effects of crowding can be directly observed by tracking the behavior of individual molecules in cells. Likewise, crowding effects in vitro have also been investigated extensively in the last years. The result is a puzzling complexity of crowding effects, as crowding can affect multiple aspects of a biophysical process, which may interfere with each other in positive or negative ways. Here, we have reviewed crowding effects on elementary physical processes and used the search of a transcription factor for a binding site on DNA as an example for a complex process, which is affected by crowding in several ways. The complexity of crowding is increased further if interactions are not only based on excluded volume, but may also be attractive or if the crowders are active particles keeping the system away from thermal equilibrium. In the cell, all these aspects (plus a great heterogeneity of the molecules) come together, so an understanding of the conditions under which biophysical processes take place in a cell will require the integration of all these different aspects of crowding.

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