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Macromolecular crowding: Sensing without a sensor

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Abstract

All living cells are crowded with macromolecules. Crowding can directly modulate biochemical reactions to various degrees depending on the sizes, shapes, and binding affinities of the reactants. Here, we explore the possibility that cells can sense and adapt to changes in crowding through the widespread modulation of biochemical reactions without the need for a dedicated sensor. Additionally, we explore phase separation as a general physicochemical response to changes in crowding, and a mechanism to both transduce information and physically restore crowding homeostasis.

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Introduction

Cells sense and respond to their environment in myriad ways. One key axis of information transfer is the modulation of the physical properties of cells by external perturbations, such as mechanical deformation, osmotic perturbations, temperature changes, or chemical alterations. It is interesting to compare two key cellular physical parameters that have widespread impacts on cellular function: tension along cellular surfaces and macromolecular crowding within cellular compartments (crowding for short). Numerous molecules that sense tension have been elucidated [1–6]; these will not be the focus of this opinion article. On the other hand, the mechanisms that sense and respond to crowding are poorly understood. This deficit is striking because we argue that macromolecular crowding is a more universally conserved property than tension. Tension manifests differently in different organisms: Plants and microbes do not possess a contractile cortex, and animal cells do not have a rigid cell wall. However, the interior of all organisms is crowded with macromolecules. Here, we focus on the sensing of crowding (Figure 1a). What are the *in vivo* consequences of crowding modulation? More generally, is there a sensor for crowding? We will discuss the possibility that cells can feel and adapt to the effects of crowding without the need for a dedicated molecular sensor; changes in crowding can alter the balance of biochemical reactions to adapt the phenotypic state of a cell to the local environment. We will also explore phase separation as a mechanism to sense crowding and a potential means to overcome excess crowding, either through active regulation or through a passive response.

What is crowding?

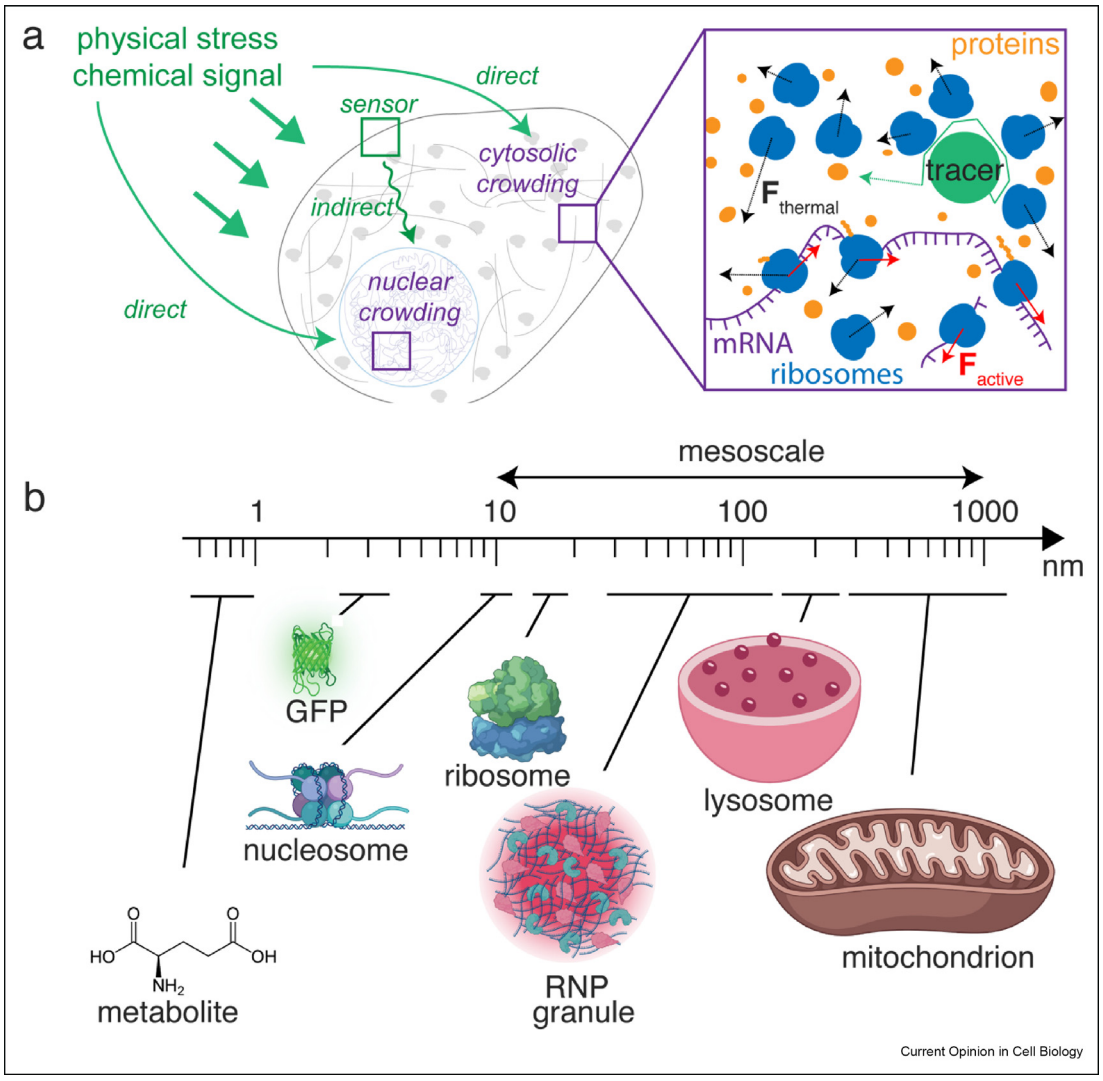
Up to 40% of a cell's volume is filled with macromolecules, at a concentration of around 200–300 mg per milliliter [7,8]. This crowded environment can inhibit molecular motion, but on the other hand can drive the assembly of macromolecules into larger structures due to an entropic effect called the "depletion attraction force" [9–11]. Crowding also has other effects, for example, changing the conformational dynamics of proteins or protein complexes [12]. However, we will simplify our discussion to consider two key effects of crowding: slowing diffusion, and favoring assembly.

Viscosity and crowding are distinguished by length-scales. We consider a solute to be a "crowding agent" if it is a similar size to the particle of interest, and to be a "viscogen" if it is substantially smaller than the particle. This is because the particle will reflect off the surface of a crowding agent, but experience viscogens as simply an increased drag. When particles are much smaller than the crowding agents in question, they are not strongly affected because they can move between gaps [13]. The majority of cytoplasmic volume is taken up by mesoscale (10–100 nm diameter) particles [8]. This means that the effects of crowding most strongly impact mesoscale particles and assemblies (Figure 1b).

Sensing crowding without a sensor

Macromolecular crowding impacts biochemical reactions both *in vitro* and *in vivo*. Theoretically, every biochemical reaction has a maximum rate at an optimal level and length-scale of crowding: crowding tends to increase reaction rates by favoring binding, but high crowding eventually inhibits reactions by reducing

Figure 1



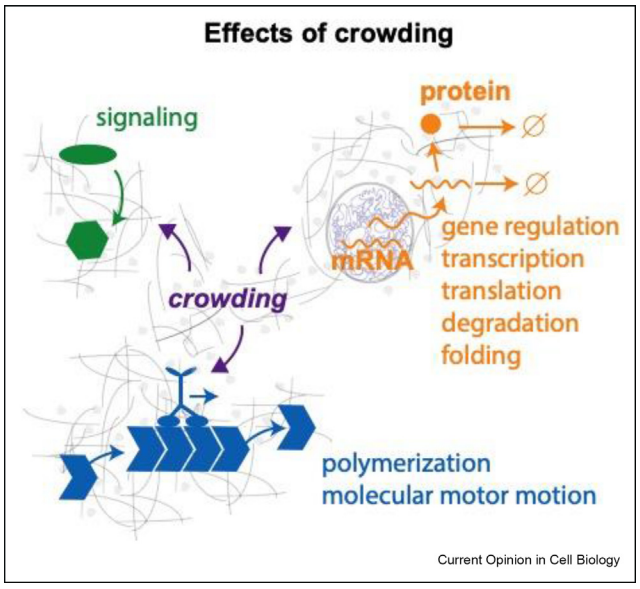
(**a**) Physical stresses can be detected either through dedicated molecular sensors or through their direct modulation of the physical properties of the cell, in particular intracellular crowding. Perturbations to intracellular crowding can alter the balance of biochemical reactions in a cell, leading to phenotypic changes "without a sensor." (**b**) The cell is crowded with molecules and proteins at different size scales. We define the "mesoscale" to be the scale ranging from tens to hundreds of nanometers.

diffusion or suppressing conformational dynamics. The relative importance of these two effects can be tuned through evolution. If the concentration of a pair of molecules is similar to the dissociation constant, their binding can be strongly influenced by crowding [14]. On the other hand, if reactant molecules bind very strongly, the effects of crowding will not be very significant. Furthermore, increasing the size of reactants will make the reaction more sensitive to the diffusion effects of crowding. Finally, reactions in a cell often involve more than two particles, increasing the number of parameters that can be tuned. As such, changes in crowding can have a distinct effect on each reaction in a cell. This altered balance of reactions can have complex consequences on cellular phenotype, impacting both cell signaling [15] and the many reactions defining biogenesis (Figure 2). In conclusion, changes in crowding can be "sensed" by its distributed effects on many biochemical reactions without the need for dedicated "sensor" molecules.

Perturbations that affect macromolecular crowding

It has become apparent that crowding is not constant in cells but can change in response to mechanical, metabolic, chemical, and genetic perturbations [16–18]. Osmotic stress alters the water content of a cell, making it a great experimental condition to study the effects of changes in crowding. A hypertonic external environment leads to water efflux, decreasing cell volume, and therefore increasing crowding, while a hypotonic external

Figure 2



Sensing crowding without a sensor: direct effects of crowding. Crowding can alter signaling or biogenesis. It can also impact the polymerization of filaments and the motion of molecular motors.

environment does the converse [19*]. Mechanical perturbations can also lead to water efflux from cells and therefore increase crowding. Crowding can also change due to a shift in the balance of catabolic and anabolic processes in cells. When cells have plenty of nutrients, the TORC1 kinase pathway increases ribosome biogenesis and decreases autophagy leading to higher molecular crowding in the cytoplasm [16]. These environmental perturbations can either increase or decrease crowding to various degrees and over distinct time-scales. We hypothesize that cells have evolved to shift their phenotype in response to these changes in crowding.

Crowding can physically modulate signaling pathways

Signaling pathways can either be inhibited or enhanced by increases in molecular crowding. Many pathways rely upon the regulated redistribution of protein molecules to new cellular locations, such as nuclear import. After strong osmotic shock, increased crowding led to a decreased rate of nuclear import of four unrelated stress-response signaling factors suggesting that signaling can be diffusion-limited [20]. On the other hand, mechanical or osmotic compression enhances the Wnt/β-catenin signaling pathway by stabilizing LRP6 signalosome formation [21**]. The increased signaling led to increased self-renewal of intestinal stem cells in organoids. In this example, increased molecular crowding potentiates signaling by favoring molecular assembly. Further investigation into which signaling pathways can be modulated by crowding will improve our understanding of cellular adaptation to physicochemical cues.

Crowding inhibits protein biogenesis

Protein biogenesis encompasses many processes, from transcription to translation, to folding and degradation. When cells are grown in confined spaces, mechanical pressure develops, which prevents cell volume increase. Continued biogenesis in the absence of volume growth then increases crowding [17**]. This increased crowding decreases protein synthesis and therefore attenuates any further crowding increase [17**]. It is still unclear which steps of protein synthesis are rate-limiting in these conditions. However, we speculate that the inhibition of protein synthesis under compression could prevent cells from becoming lethally overcrowded, and allow some essential processes to continue even though growth is stalled.

Transcription and translation are differentially impacted by crowding in vitro

The effects of crowding have been investigated in a number of *in vitro* transcription and translation assays, using synthetic crowders and viscogens such as Ficolls [22], sucrose [23,24] or dextrans [25]. Recent efforts have developed microfluidic approaches to create liposomes filled with *Escherichia coli* extract. The concentration of lysate in these "cytomimetic protocells" was then varied using osmotic perturbations [23,24]. Using this assay, the authors showed that protein production rates were relatively constant over a large range of lysate densities (from 100 mg/mL to 300 mg/mL). Theoretical fitting of the data suggests that this could be due to differential effects of crowding on translation and transcription, with optimal translation at low crowding (100 mg/mL) and optimal transcription at higher crowding (300 mg/mL) [23,24]. There are very few studies on the effect of crowding on transcription *in vivo*. One study found that hypo-osmotic stress, which decrowds the cell, is associated with increased transcription [26]. Therefore, the overall impact of crowding on gene expression is complex, and distinct results have been found in different systems. Further research in this area would be very valuable.

Compensation between ribosome number, crowding and translation rate

Ribosomes account for around 50% of the total volume of macromolecules in the cytosol [16]. Therefore, ribosomes have a dual role as both translational machinery and crowding agent. This dual role has been recently shown to have interesting consequences in *V. cholera*. Here, the *s10-spc-α* (S10) locus codes for most of the ribosomal proteins [27**]. In wild-type cells, the S10 locus is close to the replication origin (*oriC*). This leads to a higher gene dosage during most of the cell division cycle because the gene is replicated early. Experimentally moving the S10 locus further from the origin reduced the average S10 gene dosage and therefore decreased the concentration of ribosomes. However, protein production rates were not impacted because

cells with lower ribosome concentration also had lower crowding. Therefore, the decrease in translational capacity was compensated by an increase in biochemical efficiency. A similar compensation has been seen for the growth of *E. coli* under osmotic stress [28]. Thus, the dual effects of ribosomes as core factors for biogenesis and determinants of biophysical properties [16] may buffer the effects of concentration fluctuations.

Metabolic activity increases macromolecular diffusion in the crowded cell

Computational modeling suggests that protein synthesis is more hindered by high crowding than metabolism [29]. This is because protein synthesis depends on the transport and interaction of large macromolecules like mRNA and ribosomes, while metabolism relies upon the small molecules that easily diffuse past macromolecular crowders, relatively unhindered (Figure 1b.)

In general, it has been shown that metabolic activity is required to fluidize the cytoplasm at the mesoscale. Indeed, when metabolic activity is inhibited, the cytoplasm acquires solid-like properties [18,30]. Recently, it has been shown that, in the absence of metabolic activity the cytoplasm is close to a jamming transition in cells and *in vitro* and displays properties of a "fragile colloid" where the diffusivity of particles rapidly decreases with small increases in crowder volume fraction [31]. However, when the authors investigated diverse organisms (*E. coli*, *Xenopus* oocytes, or HeLa cells) in the presence of metabolic activity, they did not observe this cytoplasmic fragility. These results suggest that the properties of the cytoplasm and the requirement of metabolic activity to fluidize this material may be conserved across evolution. Theoretical work suggests that metabolism in bacteria is optimal at physiological levels of crowding [32]. Furthermore, it has been proposed that significant amounts of cellular energy are expended to fluidize the cytoplasm and increase macromolecular diffusion [32]. However, so far, there is little known about how crowding impacts the rates of metabolic reactions. Defining this relationship could give crucial insight into physical homeostasis in cells.

Crowding, transport, and filament polymerization

The fluidity of the cytoplasm requires biochemical activity and the motion of motors, but the cytoplasm affects the activity of motors in turn. *In vitro* experiments showed that crowding does not affect single kinesin motor velocity, but can significantly increase the sensitivity of cargo transport by teams of kinesins to hindering loads both *in vitro* and in *Drosophila* embryos [33**]. The increased sensitivity was explained by a model in which crowding entropically pushes together the two heads of the motor into a more compact configuration, changing the cargo detachment rate.

The dynamics of cytoskeletal filaments are also impacted by physical changes in the cytoplasm. Osmotic compression decreases both microtubule dynamics in *Schizosaccharomyces pombe* and mammalian cells [34*]. Polymerization and depolymerization rates both changed equally and *in vitro* experiments showed that viscosity changes were sufficient to recapitulate similar changes in microtubule dynamics. Other *in vitro* work showed that microtubule polymerization rates are increased by crowding and decreased by small viscogens [35]. The actin cytoskeleton is also impacted. *In vitro*, crowding drives actin bundling [36], but decreases filament polymerization rates [37]. Crowding also enhances polymerization of the bacterial cytoskeletal MreB filament [38]. Thus, we need to consider both crowding and viscosity to fully understand the physical regulation of cytoskeletal dynamics.

Since cytoskeletal and motor activity can help facilitate particle motion through crowded environments, we speculate that it may be adaptive for increased crowding to potentiate these activities. For example, this could help cells to more effectively "self-agitate" to maintain fluidity when they are compressed.

Phase separation as a putative sensor of crowding

Phase separation of biomolecules has gained considerable interest in the past decade owing to its potential to confer rapid regulation. Phase separation is the spontaneous separation of a well-mixed solution into two or more phases of differing densities. This is a fundamental physicochemical principle that appears to manifest in many biological systems. Phase separation has been extensively reviewed recently [39]. Here, we will focus on the relationship between phase separation and macromolecular crowding [40]. Increased crowding can favor phase separation both *in vitro* and *in vivo* [16,41]. On the other hand, very high crowding, or solid-like networks like chromatin in the nucleus, can prevent droplet fusion and therefore frustrate the growth of condensates [42,43]. Therefore, phase separation can transduce changes in molecular crowding into mesoscale changes in cellular organization.

Recruiting signaling molecules [44] and biosynthetic pathways [45,46] into condensates can impact their activity. For example, recruiting kinases into synthetic condensates accelerated phosphorylation events both *in vitro* and *in vivo* [47**]. Furthermore, phosphorylation within these condensates increased when cytoplasmic crowding was increased while phosphorylation outside condensates was decreased. We hypothesize that phase separation is a physicochemical sensor of crowding that can be transduced to biochemical activities in myriad ways (Figure 3).

Phase separation and transcriptional regulation

Condensation of several transcriptional regulators may play a role in the control of gene expression [48]. RNA Polymerase II can form condensates, and is thought to form coacervates with transcription factors and other transcriptional machinery [49–51]. Similarly, the epigenetic state of the pathogenic yeast *Candida albicans* is determined by transcription factors that condense on super-enhancers to self-sustain a transcriptional positive feedback loop [52]. The mechanosensor YAP/TAZ can form condensates on super-enhancers to promote gene transcription [53,54]. YAP/TAZ phase separates in response to osmotic compression, and therefore may be responding to increased crowding. It seems likely that changes in crowding will impact the transcription of many other genes by modulating transcriptional condensates.

Phase separation to de-crowd the cell

Excess crowding is rate limiting for growth [17**]. Therefore, in certain situations, it may be adaptive to decrowd the cell. We foresee three non-mutually exclusive possibilities to reduce crowding: increasing cell volume, condensing a subset of macromolecules to create space elsewhere, or decreasing the amount of material in the cell.

As discussed above, hyperosmotic stress shrinks cells leading to elevated crowding. The purpose of osmoregulatory pathways is to adapt cell volume to de-crowd the cell. A recent study in mammalian cells found that the crowding-induced phase separation of the apoptosis signal-regulating kinase 3 (ASK3) leads to its inactivation [55*]. ASK3 inactivation under osmotic stress is necessary both for cell volume recovery and to prevent cell death [56]. Similarly, the WNK1 kinase was recently found to phase separate in response to increased crowding. Phase separation of WNK1 leads to a change in kinase activity and triggers cell volume increase [57**]. Thus, increased crowding leads to phase separation of a kinase, which then triggers a chemical signaling cascade that leads to volume increase, thereby restoring normal crowding.

Phase separation could also directly de-crowd the cell. For instance, P-bodies and stress granules are condensates of mRNA and proteins that can regulate the translation and degradation of the transcripts [41,58]. However, recent work has identified a putative additional role for these condensates: de-crowding the cytoplasm. Polysome collapse and condensation of mRNAs increase the fluidity of the cytoplasm [59]. Here, the temporary sequestration of biomolecules may decrowd the dilute phase or remove molecules that would otherwise form tangled networks in the cytoplasm.

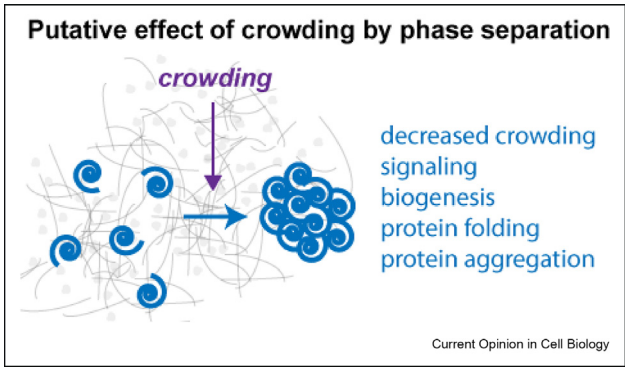
Autophagy plays a critical role in the regulation of crowding by reducing the concentration of proteins and mRNAs [16]. In yeast, autophagosomes can also degrade biomolecular condensates [60]. Autophagy is triggered by phase separation of Atg proteins [61]. Thus, high levels of crowding could drive condensation, trigger autophagy and thereby reduce crowding.

Concluding remarks

Specific mechanisms have evolved to sense and respond to changes in crowding. Phase separation is one mechanism to sense crowding, as in the examples of WNK kinase [57] and the YAP/TAZ transcription factors [53,54]. These specific sensors of crowding may be distinct across evolution and will require extensive work to be elucidated.

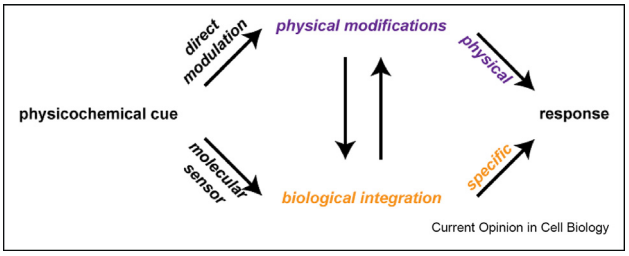
On the other hand, we postulate that changes in crowding are likely to impact hundreds or thousands of cellular biochemical reactions with far-reaching effects on signaling, biogenesis, and the material properties of the cell without the need for a specific "sensor" (Figure 4). This widespread modulation may represent a primordial mechanism of cellular homeostasis.

Figure 3



Phase separation can be enhanced by crowding. Increased phase separation could lead to specific responses to crowded conditions.

Figure 4



Physicochemical cues can trigger sensors, but also modulate the biophysical properties of the cells. Modulation of crowding can be directly sensed by the cell, for instance through phase separation, but also have indirect consequences by modulating signaling or biogenesis. The overall response to the cue incorporates both widespread physical effects and specific responses.

In *eukaryotes*, changes in crowding are likely to impact other subcellular compartments. We did not discuss these effects in detail because less is known. For example, crowding in the nucleus is likely to be crucial for the kinetics of processes that depend on mesoscale machinery, such as transcription or DNA replication. However, the main mesoscale crowders in the nucleus have not been well defined. We speculate that chromatin and RNA molecules are likely to be very important. In yeasts and mammalian cells, nuclear crowding and cytosolic crowding seem to be coupled through colloidal osmotic balance [17,19,62,63], such that hyperosmotic stresses lead to proportional increases in crowding in both compartments. Chromosome compaction responds to changes in crowding [64,65], and chromatin compaction increases upon osmotic stresses [66]. The chromatin network also causes elastic confinement that inhibits droplet fusion during phase separation [42]. The nucleolus, which is the largest phase-separated structure of the nucleus, could also potentially modulate the density of the nucleus; it was recently found that nucleolar density increases under hyperosmotic shock [66]. Together, these results suggest that changes in crowding are likely to have widespread impacts on nuclear processes. The same is likely to be true within other compartments such as the endoplasmic reticulum and mitochondria.

The impacts of changes in crowding on cellular phenotypes are only just beginning to be discovered, partly because it is challenging to disentangle the roles of signaling pathways and physical effects. However, it is likely that crowding is crucial for many aspects of physiology. Failure of homeostatic responses to changes in crowding is also likely to be important in pathology. For example, aberrant phase separation is increasingly associated with disease [67], and crowding can influence phase separation. Therefore perturbations to crowding could contribute to disease both by driving aberrant phase separation and shifting the overall balance of reactions in a cell.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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