Review Article

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Quest for space: Tenacity of DNA, Protein, and Lipid macromolecules in intracellular crowded environment

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Abstract: The biochemical processes in the cellular milieu involving biomacromolecular interaction usually occur in crowded and heterogeneous environments, impacting their structure, stability, and reactivity. The crowded environment *in vivo* is typically ignored for experimental investigations since the studies get complex due to intracellular biophysical interactions between nucleic acids, proteins, cellular membranes, and various cations/anions present in the cell. Thus, being a ubiquitous property of all cells, studying those biophysical aspects affecting biochemical processes under realistically crowded conditions is of prime importance. Crowders or crowding agents are usually exploited to mimic the in vivo conditions on interacting with such genomic species, revealing structural and functional changes resulting from excluded volume and soft interactions. In the last few years, studies including crowders of varied sizes have gained attention concerning the consequences of crowding agents on biomolecular structural transitions and stability. This review comprehensively summarizes macromolecular crowding, emphasizing the biophysical effects and contribution of soft interactions in the heterogeneous cellular environment.

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Introduction

Structural features and stability of biomacromolecules like DNA, RNA, proteins, and lipids (cellular membranes) are imperative aspects of cell life. DNA and RNA are genetic materials that store genetic information, making them crucial for innumerable biological activities. Synthetic DNA and RNA oligonucleotides hold promise for diagnostics and therapeutics, which include human gene regulation [1,2], gene expression analysis [3,4], and target molecule sensing [5,6], as base pairing permits highly selective hybridization with a target sequence. However, it is difficult to predict the stability of nucleic acid and protein structures solely based on the sequence composition, as this characteristic is highly reliant on the environment of the solution in which it exists.

Biomacromolecules constitute a sizable portion of living cells, cultivating an inherently compact intracellular environment. Compared to standard *in vitro* conditions, the interior environment of the cell is clustered with macromolecules, which retain 5–40% of the total cellular volume. Although the amounts vary according to the cell type, differentiation stage, and cell volume, eukaryotic cells contain 50–400 mg mL^{-1} biomolecules in the cytoplasm, 100–400 mg mL^{-1} in the nucleus, 100–200 mg mL^{-1} in nuclear organelles, and 270–560 mg mL^{-1} in the mitochondrial matrix. According to reports, *Escherichia coli* contains 300–400 mg mL^{-1} of biomolecules overall, with 200 mg mL^{-1} of protein, 75 mg mL^{-1} of RNA, and 10–20 mg mL^{-1} of DNA, depending on the stage of development. Crowding occurs not only intra-cellularly but also extracellularly in tissues; even blood plasma contains 80 g L^{-1} of protein, which is enough to induce large-volume crowding effects [7–13]. This crowded extracellular milieu is termed macromolecular crowding. Since no specific molecular species exist at high densities, the environment is

seen as crowded but not overly concentrated [14]. This crowded milieu affects biomolecular structure, stability, and functionality through phenomena such as excluded volume effects, soft interactions, and hydration perturbations. Crowding is a ubiquitous cellular property requiring in-depth exploration to understand its implications for biomolecular activity.

In vitro studies utilizing various crowding agents are aiding in filling the gaps in our comprehension of the processes involving various biomacromolecules and their *in vivo* activities, as shown in Figure 1. The term macromolecular crowding is typically used to describe crowding brought on by large molecules, whereas molecular crowding refers to crowding caused by small molecules. However, these terms are frequently used interchangeably [15]. The kinetics of interstitial bulk-like water molecules and those at the crowder/water contact are unaffected by molecular and macromolecular crowders in less crowded environments. Although macromolecular crowding has only a small crowding effect on the water at the crowder/water interface and has no effect on the bulk-like hydration dynamics in densely crowded media, interstitial water dynamics strongly rely on molecular crowding in such media [16]. It is speculated that weak interactions with no profound influence might become more prominent in crowded conditions. The intracellular environment drives many cellular activities, including intracellular phase separation, molecular compartmentation, glucose metabolism, tumor development, and susceptibility to illnesses with aging [17–23]. In recent decades, more attention has been paid to crowding to access the potential behavior of biomacromolecules in cell-mimicking environments. Interestingly, a recent review article by Alfano et al. has described the molecular crowding paradigm from polymer physics to a biological perspective [24].

Despite significant advancements in our knowledge of macromolecular crowding, critical gaps persist in our understanding of the underlying biophysical mechanisms. Although excluded volume effects are still well-studied and characterized, the role of hydration dynamics and soft interactions is underexplored, particularly in heterogeneous cellular environments. Furthermore, the contribution of macromolecular crowding to lipid dynamics and membrane organization is less studied than that of DNA and proteins. Additionally, while *in vitro* studies using crowding agents have provided valuable insights, translating these findings to complex *in vivo* systems, where spatial and temporal variations in crowding occur, remains a significant challenge. This review tries to fill these gaps by providing a complete overview of macromolecular crowding caused by various polyethylene glycols (PEGs), Ficolls, and dextrans and their effects on biomolecules, with a focus on DNA, proteins, and lipids.

Biophysical consequences of macromolecular crowding

Macromolecular crowding conditions utilize the high concentrations of various cosolutes to investigate chemical

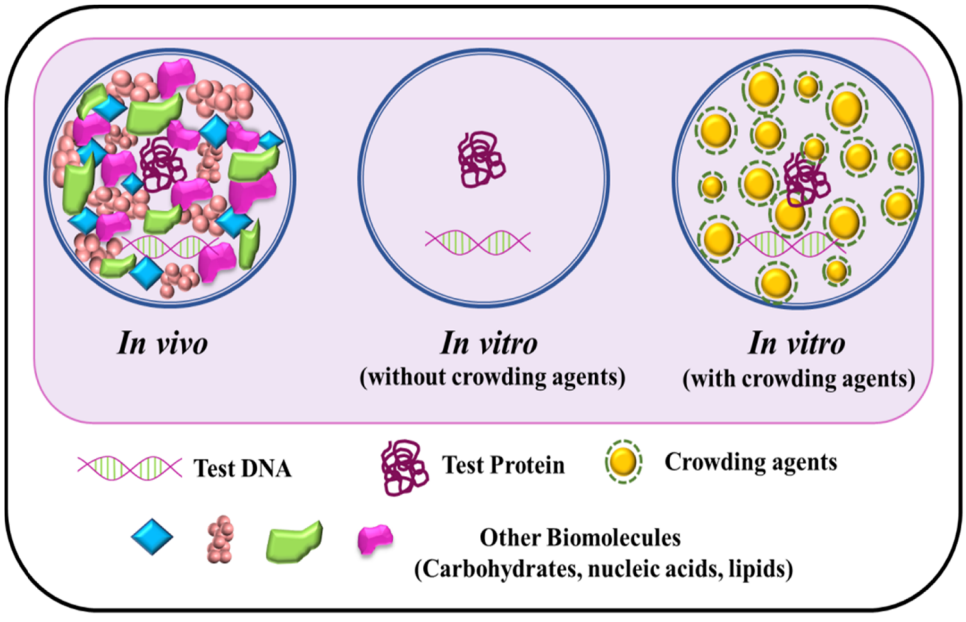

Figure 1: Schematic representation of DNA or proteins under different conditions.

and biochemical reactions by altering kinetic and thermodynamic parameters. It has been depicted that molecular crowding works based on two important principles – excluded volume effect (repulsive hard interactions) and chemical effects (attractive soft interactions like salt bridging, H-bonding, hydrophobic effect, etc.) [25]. The properties of the medium of cellular processes are altered by the cells' highly concentrated and limited environment. The following section discusses the impact of cosolutes on various solution properties.

Excluded volume

Under ideal conditions, molecules are considered to have no volume occupancy, as these are generally represented as dots. The term "excluded volume" refers to the volume occupied by a molecule not available to the background molecules [17,26]. It is a consequence of macromolecular crowding. The area inhabited by macromolecules and organelles is restricted from other components by steric hindrance, establishing an excluded volume [18]. It originates from molecules getting spatially constrained in a small volume. Crowding also limits the amount of solvent accessible for other molecules in the solution, significantly impacting the structure and stability of biomacromolecules. When macromolecules occupy a significant portion of the total volume and the size of the probe species is equal to or larger than the size of the crowder species, the effects of excluded volume on transfer-free energies become apparent [27]. In a recent study, Minton showed that the activity coefficient of a target molecule exhibits an exponential increase with fractional volume occupancy, highlighting a nonlinear dependence on molecule concentration [28]. Controlling the level of osmolality, the intracellular environment helps to regulate the cell volume, which is believed to be crucial for cell growth and proliferation [29,30].

The thermodynamic activities of dissolved molecules are altered by steric effects, consequently affecting the kinetics and thermodynamics of biological reactions. Reactions that result in volume reduction are favored because the system's free energy increases as the configurational entropy of the system molecules remains constrained [31]. However, it is also hypothesized that at low concentrations of macromolecular crowders, the soft interactions (which are enthalpically driven) have a more substantial effect than the excluded volume [32]. Since the activity coefficients of the molecules in the system vary because of the excluded volume, the chemical potentials of the molecules in the system also change. Theoretical and experimental results show that the excluded volume effect on the activity coefficient depends on molecule sizes and is significantly nonlinear with molecule concentration [27,31].

The excluded volume effect impacts the reaction kinetics by enhancing effective reactant concentration and favors biomolecular interactions that stabilize native structures. Typically, it enhances the biomolecular interactions; however, not all the interactions benefit from crowding. For example, studies on SARS-CoV-2 nucleocapsid protein demonstrated that small PEG molecules promote expansion, whereas large PEG molecules induce disordered region collapse [33]. This complexity is evident in various studies on the structure dynamics of protein, underscoring the necessity of a thorough understanding of cellular environments and their effects on biomolecular behavior.

Osmotic pressure

A considerable portion of cells is water that makes up roughly 70% of the total volume, i.e., approximately 40 M in a normal cell. In contrast, other molecular species make up less than 1 M. Biomolecules and immobile cellular components are hydrated by water layers, which reduces the number of free water molecules. Volume fraction and free water content fluctuate *in vivo* during the cell cycle depending on cell conditions [34]. Moreover, water is integral to molecular stability dynamics, particularly in crowded cellular environments. In crowded environments, the hydration layers surrounding biomolecules undergo significant perturbation, as demonstrated by Sen and co-workers [35,36]. These studies reveal that the entropic gain may lead to destabilizing entropic effects and entropy–enthalpy compensation despite the favorable enthalpic change on perturbation in hydration layers from crowder interaction. This relationship between hydration dynamics and macromolecular stability highlights the need to consider water-mediated interactions alongside traditional crowding effects.

Even though small hydrophilic compounds do not produce a significant amount of excluded volume, these metabolites are frequently found in living cells and alter the behavior of water molecules. Osmolytes significantly alter the properties of biomolecules by altering their hydration [37]. Different studies demonstrate that crowding has an impact on osmotic pressure. Due to its significance, several researchers have explored the effect of osmotic pressure on the properties of biomolecules. The osmotic stress phenomenon given by Parsegian et al. is commonly used to investigate the effects of osmotic pressure on biomolecules.

They employed the Gibbs–Duhem relation, osmotic stress, hydration, molecular crowding, and other factors to explain the indirect effects of cosolutes on the properties of biomacromolecules [38]. The osmotic pressure studies demonstrated the stability of nucleic acid and protein structures under osmotic pressure, as well as the hydration changes that take place before and during a reaction.

Various other physical properties of the intracellular solution are influenced by macromolecular crowding and highly concentrated cosolutes. Crowding also impacts solute transport by raising the medium's effective viscosity [39]. For example, an intracellular solution has different dielectric properties than a diluted solution. Yeast cells are thought to have a dielectric constant of 50 or even lower, which is significantly lower than the value of pure water, around 80 [40].

Soft interactions

Macromolecular crowding significantly impacts structural stability through intricate mechanisms involving both direct and indirect interactions. Traditionally, excluded volume effects were thought to enhance stability by restricting conformational freedom. However, recent studies reveal that soft interactions, including transient, weak interactions between crowders and biomolecules, can counteract this stabilization. For instance, these interactions may lead to partial unfolding or destabilization, as shown in studies on crowding-induced enthalpic and entropic contributions [41–43].

The interplay between soft interactions and other crowding aspects, such as excluded volume effects and hydration dynamics, is essential for understanding biomolecular behavior under physiological conditions. Recent research underscores how the binding of soft crowders like PEG induces structural and functional changes in enzymes, exemplified by human arginase-I, where PEG was observed to influence its catalytic site, leading to reduced function at higher concentrations [44].

Chemical phenomena such as van der Waals forces, electrostatic interactions, and hydrogen bonding can also have an impact on biomolecular stability, binding interactions, and enzyme kinetics in the densely populated cellular milieu. Charged crowders or biomolecules may influence stability through repulsion or attraction. For example, repulsion between negatively charged surfaces can destabilize native structures. At the same time, attractive interactions between crowded molecules, or between crowder molecules and tracer molecules, can partially counteract the effects of excluded volume altering the solubility and stability of macromolecules, especially proteins [45,46]. Hydrophobic regions of biomolecules may also interact with crowders, either stabilizing the native state or leading to aggregation. These notably weak interactions are primarily enthalpic in nature and become more significant at higher concentrations of the crowders, where the spatial proximity of the molecules amplifies such interactions. Processes like protein folding and macromolecular association generally entail a decrease in solvent-accessible surface area as the reactants transition into the products. Such transformations become energetically or thermodynamically favorable when repulsive interactions are mitigated [25]. The collective effects of macromolecular crowding ultimately dictate the functional outcomes in cellular environments. Figure 2 attempts to show the effect of the excluded volume and soft interactions on the structure and diffusion of the biomacromolecules. In some cases, excluded volume effects and soft interactions (hydrophobic interactions) exhibit synergistic effects to stabilize the native structures. In other cases, hydration dynamics or unfavorable soft interactions may have a competitive effect in the presence of excluded volume and destabilize the native structures. The cellular systems are highly dynamic in nature and require a delicate balance between excluded volume, soft interactions, and water dynamics. Integrating these crowding effects under physiological conditions remains ambiguous and needs further investigation.

Crowding agents to mimic *in vivo* crowded environment

Since experimental and analytical studies of biomolecular interactions involving DNA or proteins within a living cell are challenging, *in vitro* experimental techniques are frequently used. These techniques incorporate cosolutes of various molecular weights or buffered solutions with high background molecule concentrations, known as crowding agents. For these additives to act as crowding cosolutes, prerequisites such as water solubility, inertness to target molecules (DNA, RNA, or proteins), and ease of availability must be met [15].

Non-ionic polymers like PEG and polysaccharides like Dextran and Ficoll (Figure 3) are widely used cosolutes to mimic macromolecular crowding conditions. The fact that they have different molecular weights and are highly soluble in water makes them the dominant crowding agents. Furthermore, a protein BSA is used as a crowding agent in biological operations to replicate the effects of

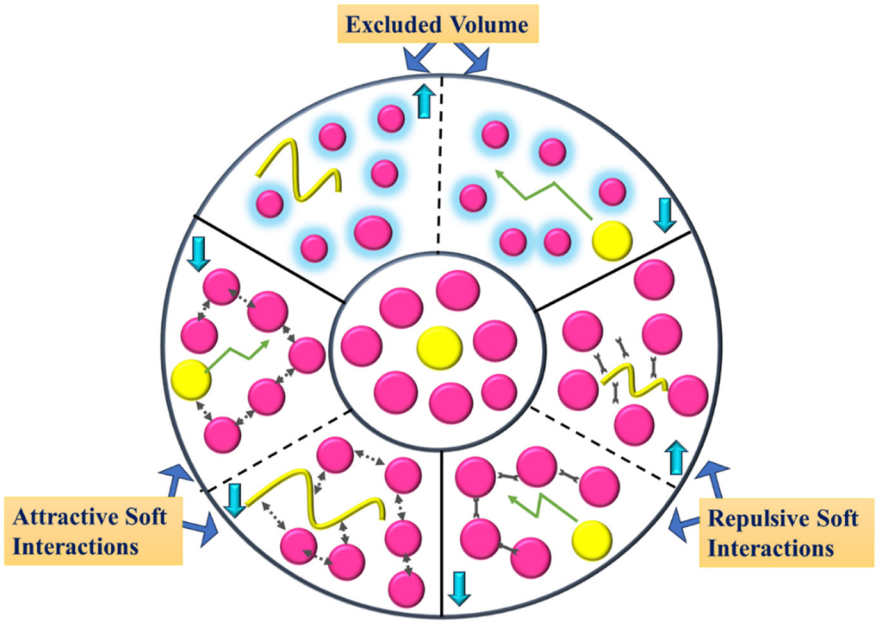

Figure 2: Pictorial representation of effects on volume exclusion and soft interactions due to molecular crowding. Each background molecule (pink color) forms an exclusion region for a given probe (yellow color), which is shown by the shaded blue circle. The aqua-colored arrows (present inside the circle) represent the increase/decrease in the structure stability (yellow) and diffusion (green arrows) of the biomacromolecules. *Some exceptions are always there in different crowding agents.

high concentrations of proteins. Nevertheless, it exhibits negative charges on its surface, perhaps interacting with DNA and proteins [47]. Small crowding agents like glycine betaine, acetamide, trimethylamine-N-oxide, etc., have also been used for *in vitro* studies [48–51]. Under stress conditions, these osmolytes protect protein structures [52]. The impact of crowding on the equilibria and kinetics of biological activities has been well studied experimentally using these crowding solutes. PEGs and polysaccharides are frequently used as macromolecular crowding reagents, even though they can cause precipitation and preferentially interact with proteins or nucleic acids [53,54]. None of

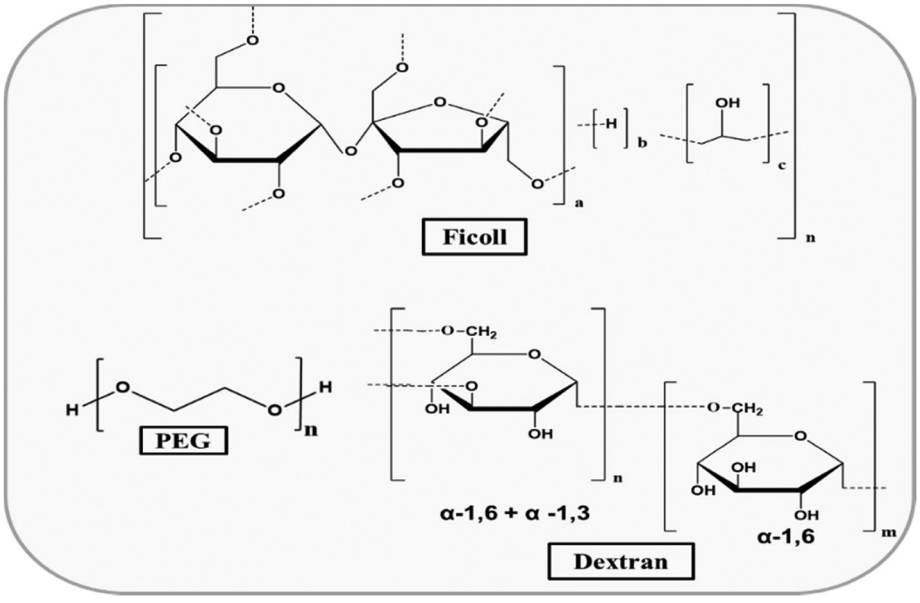

Figure 3: Different macromolecular crowding agents used in *in vitro* studies.

these agents perfectly replicate the heterogeneous and dynamic nature of the cellular interior, which includes a wide variety of macromolecules with diverse shapes, sizes, and interactions. These crowding agents lack the specific interactions and binding affinities present in biological systems, which can lead to oversimplified models. Mixed crowding conditions are also used to mimic such environments [55–57].

The larger PEGs create a region inaccessible to other molecules, increasing the viscosity of the solution. The excluded volume effect, which increases the biomolecule's thermodynamic activity, may increase their association rates; however, when the reaction probability is close to unity, the increased viscosity, which reduces diffusion rates, overcomes the excluded volume effect [58,59]. Small solutes, on the other hand, do not operate as barriers but rather alter the solution's properties. In particular, ethylene glycol and small PEG molecules lower water activity and increase osmotic pressure (osmotic stress). As a result, the molecular environment differs significantly and can be influenced by the type and size of solutes utilized in the study. However, the primary objective of this review is to explore how macromolecular crowding agents affect the stability and structure of biomacromolecules.

Structural status of nucleic acids, proteins, and lipids in crowded conditions

Nucleic acids

Nucleic acids are capable of deciphering complementary sequences, which leads to the formation of double helical structures through the generation of Watson–Crick base pairs. Hoogsteen base pairing generates non-canonical structures like triplexes and tetraplexes [60]. Previous works have reported a significant effect of crowding on the structure and behavior of nucleic acids. Effects of macromolecular crowding on the compaction, extension, and kinetics of DNA and protein–DNA interactions have been reviewed well [61]. However, the consequences of macromolecular crowding on the structural characteristics of nucleic acids are currently a trending topic in the study of physiology and metabolism *in vivo*. Large and small oligonucleotides have been employed in researching nucleic acids with crowding agents of different molecular weights. Literature is rich in reports deciphering the role of crowding agents in determining the structure and stability of DNA. It has been found that crowders suppress thermal fluctuation by restricting the movement of individual molecules in the cell. During DNA melting, the crowders block the bubble propagation. However, further strand fluctuation displaces the crowders from the original sites and opens the previously crowded site, and thus, double-stranded DNA transforms into a single strand. Hence, the excluded volume by biomolecules and the chemical interactions play a vital role in determining the stability of DNA molecules [62].

Canonical forms

Duplex, or the double helical structure, is the predominant form of DNA in the genome. The solution conditions like hydration, viscosity, and dielectric constant affect the stability of nucleic acid structures. However, hydration is considered as a critical factor in regulating the thermodynamic stability of DNA duplexes, while viscosity and dielectric constant have only a minor effect [63]. Initially, 10 wt% solution of PEGs (molecular weight = 4×10^3 and 2×10^4) raises the melting temperature (T_m) of poly(I) poly(C), a homopolymer RNA duplex with polyinosine and polycytidine strands, by 1.8–2.0°C, suggesting an increase in stability accounted due to volume exclusion effect [64]. Similarly, the stability of poly(dA)·poly(dT) duplex increases in the presence of PEG solutions as the T_m rises by approximately 5°C at 19 wt% PEG-8000, but small cosolutes decrease the duplex stability [65,66]. The T_m of 5′-dATGCGCAT-3′ was decreased by 11.8 and 1.5°C in the presence of 20 wt% of PEG-200 and PEG-8000, respectively. Interestingly, with a decrease in NaCl concentration from 1 M to 400 mM, the T_m increased by 2.8 and 3.3°C in the presence of PEG-8000 and PEG-200, respectively [67]. However, the stability of DNA duplex depends not only on the molecular weight and the concentration of cosolutes but also on the nucleotide length. The T_m of 8-mer sequence (5′-ATGCGCAT-3′) and 30-mer sequence (5′-A27GCG-3′/5′-CGCT27-3′) increase by approximately 15 and 10°C respectively in the presence of PEG-200. For short duplexes, the destabilization was explained in terms of reduced water activity [68].

According to Gu et al., the kinetics of duplex formation get altered by the addition of small cosolutes, but the rate constants were unaffected in the presence of PEG-8000 [69]. Similarly, the kinetics of 12- and 16-base-pair-long probes does not change significantly *in vitro* under the crowded environment of large Dextrans and Ficoll [70]. RNA duplexes and RNA–DNA hybrids also get destabilized in the presence of small cosolutes, which was in accordance with the previous studies of DNA duplexes [50,70–74]. However, crowding agents of high molecular weight, i.e., more

than the molecular weight of PEG-1000, tend to stabilize the folded structure, which follows the excluded volume effect because large cosolutes tend to cause more steric hindrance and favors the reactions that decrease net volume and thus increase T_m [75]. It is also important that small cations like Na^+, K^+, Ca^{2+}, and Mg^{2+} can affect the molecular crowding in the solution. Studies show that PEG as a crowding agent enables Na^+ to compact double-stranded DNA more efficiently than K^+ [76]. For the prediction of thermodynamic stability of duplexes, a nearest neighbor (NN) model has been developed, which predicts the thermodynamic parameters (like $\Delta G°$, $\Delta H°$, $\Delta S°$, or T_m) without experiments. The basis of this prediction is the assessment of a base pair's stability through its interaction with its NN base pair. The investigations that used short synthetic sequences yielded more information. The advantage of employing oligonucleotides is that in a two-state transition, a duplex with a short nucleotide length like 10–20 base pairs is likely to melt into a single-stranded state, allowing accurate determination of the thermodynamic parameters. This method can predict the duplexes under molecular crowding conditions [77].

Non-canonical forms

Apart from the Watson–Crick base pairing, a wide range of stable noncanonical forms of DNA also exists. Figure 4 depicts structural polymorphism in nucleic acids resulting in the development of left-handed helix, triplex, G-quadruplex, and i-motifs based on the sequence and chemical environment [78–80]. A partial change in Watson–Crick base pairing generates these structures in genomic DNA. Because of their existence in regulatory control regions of the genome, these structures are frequently related to gene regulation, mutagenesis, diseases, and protein interactions [81–86]. The crowding conditions allow us to interpret the change in kinetics or structure of these non-canonical forms *in vivo*.

Z-form or left-handed double helix form

Unlike conventional right-handed B-DNA, Z-DNA forms a left-handed double helix having a zigzag pattern with base pairs perpendicular to the phosphate backbone. The crystal structure of DNA fragment d(CpGpCpGpCpG) revealed the presence of the first Z-DNA ever [87]. In the transcribed part of the genome where alternating pyrimidine and purine bases are present, DNA tends to adopt Z-form *in vivo* under a physiological environment. Z-form has the potential for gene regulation, and it is intrinsically linked with gene instability [88]. The formation of Z-form and B-form depends on the solution conditions. *In vitro* studies showed that the B-form of DNA occurs at low or moderate salt concentrations, whereas the Z-form is prone to be found at high salt concentrations [89]. It has long been known that water plays a significant role in altering the conformational states of nucleic acids, and a variety of physio-chemical techniques have been employed to study the interaction between

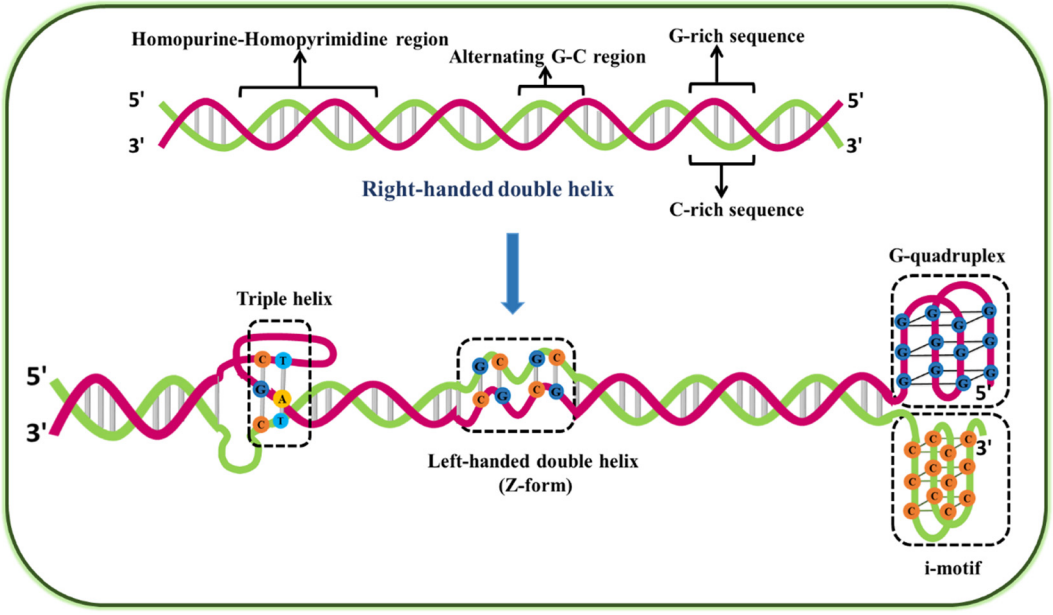

Figure 4: Illustration of the triple helix, G-quadruplex, left-handed double helix, and i-motif structures that arise in sequence-specific areas of genomic DNA.

nucleic acids and water [90]. Accordingly, the Z-form is believed to occur in less hydrated locations [91]. Under crowded conditions of PEGs, glycerol, or other hydroxyl groups containing cosolutes, the energy barrier for the Z-form was less at modest salt concentrations. The osmotic pressure impact favoring the dehydration reaction is probably responsible for forming the Z-form of DNA in these conditions [92]. This hypothesis accorded well with the earlier findings of Westhof [91].

Triple–helical structure

Hoogsteen and reverse-Hoogsteen base pairing results in triple–helical structure formation when the single-stranded triplex-forming oligonucleotide cohered to the major groove of a duplex structure having polypurine polypyrimidine stretches. The binding of the third strand to the duplex structure arises because of the presence of residual hydrogen bonding sites in the structure after Watson–Crick base pairing. In DNA, Hoogsteen and Watson–Crick base pairing is generally accompanied by dehydration and hydration, respectively. The decrease in water activity due to macromolecular crowding conditions is responsible for the stabilization of the DNA triplex structures [65,93]. Considering the volume exclusion effect, PEGs of high molecular weight were used to stabilize poly(dT)poly(dA)poly(dT) triplex under crowded conditions [66]. In the presence of 15% (w/v) of PEG, the T_m of triplexes T_{18}*$(AT)_{20}$, $T_{17}I$*$(AT)_{20}$, and $T_{16}I_2$*$(AT)_{20}$ was found to be increased by 15–16°C, possibly favored by enthalpic changes [94]. Cations like K^+ or Na^+ also influence triplex stability. Crowding cosolutes with different salt concentrations and temperatures could effectively compensate for changes in environmental conditions. Under molecular crowding conditions, the effect of K^+ on triplex DNA stability has also been reported. In the presence of the crowding agent PEG, the formation of triplexes was stabilized at low concentrations of KCl [95]. Similarly, the divalent cations (Ca^{2+} and Mg^{2+}) and molecular crowding buffers have been shown to stabilize G-triplex at physiologically relevant temperatures [96].

G-quadruplexes and i-motifs

Among the four nitrogenous nucleic acid bases, guanine shows peculiar behavior as it is capable of forming G.G base pairs by Hoogsteen H bonding. The self-association between four coplanar guanine bases results in the formation of diverse intramolecular or intermolecular G-quadruplexes. The genomes of diverse organisms possess a significant number of guanine-rich regions with the potential to generate G-quadruplexes [97–100]. For the last two decades, countless studies under varied solution conditions have been reported on the structure and stability of G-quadruplexes. The structural polymorphism of G-quadruplex is mainly influenced by solution conditions like metal ions, loop size, the presence of cosolutes, and other factors [101–104]. Herein, we specifically discuss the effect of cosolutes on the quadruplex structure.

The G-quadruplex structure is stabilized by the crowding agent PEG-200, supporting earlier findings that Watson–Crick base pairing is destabilized in the presence of cosolutes whereas Hoogsteen base pairing is stabilized. Human telomeric G-4 DNA gets dehydrated throughout the folding process, influencing its stabilization by decreasing water activity induced by the crowding reagents [105]. Hydration depends on the folding of the structure, loop size, G-quartets, type of cosolutes, and metal ions present in the solution [106–109]. The effect of K^+ and PEG concentration was investigated, concluding that stable G-quadruplexes could only form under the crowding condition with PEG at concentrations near the physiological concentration [110,111]. The crowding effect on quadruplex changes with variation in guanine quartets' topology or the type of cosolutes. Figure 5 depicts that PEG (average MW = 300) can alter the G-quadruplex topology of $d(G_4T_4)_3G_4$ and $d(G_4T_4G_4)_2$ from anti-parallel to parallel conformations [112]. Quantitative analysis of thermodynamic parameters showed that G-quadruplexes exhibit entropy and enthalpy changes due to excluded volume by cosolutes and the chemical interaction between DNA and the solutes, respectively.

The structural transition mediated by crowders was observed in human telomeric quadruplexes. The presence of PEGs brings a dynamic conversion of two quadruplex units to parallel conformations stepwise [113]. Furthermore, reports showed that in the presence of PEGs or Ficoll with K^+ ion buffer, human telomeric quadruplex structure exists in mixed conformations with a decrease in stability predominantly due to the dehydration factor and not due to the volume exclusion effect [114,115]. Earlier reports have shown that small cosolutes such as betaine, ethanol, acetone, and dimethylformamide maintain the quadruplex structure. This resulted from modifications to the solvent's dielectric characteristics [116]. Kan et al. showed that the competition between G-quadruplex and duplex formation intensified in environments with low salt concentration levels [117]. Hence, these studies provide the physical basis of G-quadruplex formation in the genome and their existence under an *in vivo* crowding environment.

The intercalated $C.C^+$ base pairs can generate another distinct tetraplex structure named i-motif in an oligomeric

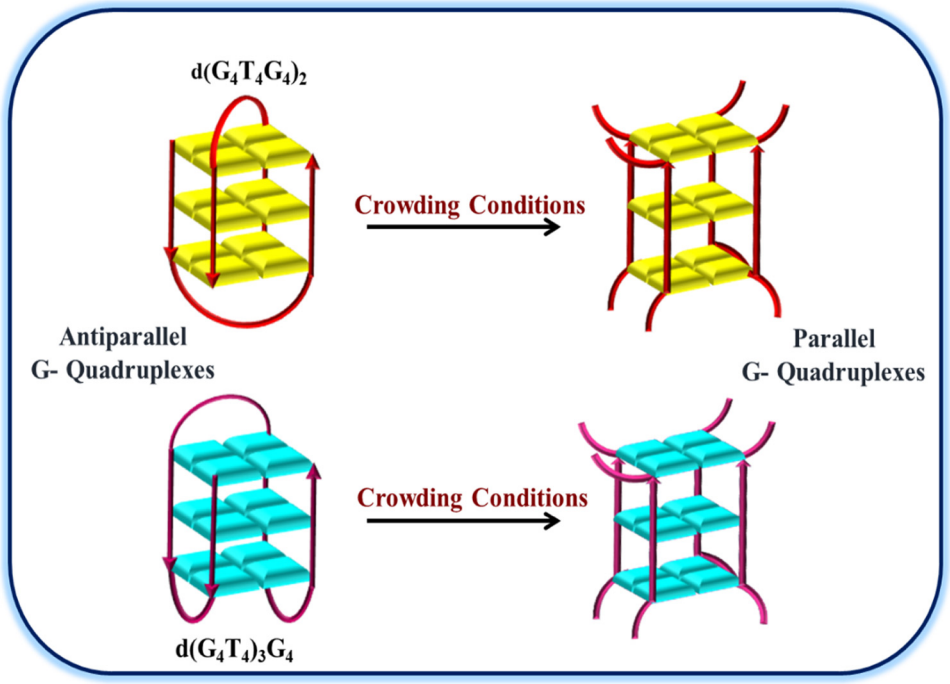

Figure 5: Diagrammatic representations of the structural change caused by molecular crowding from the antiparallel to the parallel G-quadruplex of $[d(G_4T_4G_4)]_2$ and $d(G_4T_4)_3G_4$ [112].

cytosine sequence and demand protonation of the cytosine (C) residues. The CCT repeat sequences from an established repetitive region in the human genome create the i-motif at acidic pH but hardly at neutral pH. Sugimoto et al., on the contrary, reported the first instance of an i-motif structure at neutral pH made from unstructured triplet repeat oligomers in the presence of PEGs with varied molecular weights. It was hypothesized that the generation of the i-motif resulted from the decreased pK_a value of cytosine in crowded conditions [118]. Studies from other groups also reinforce the fact that the crowded solution condition facilitates the c-MYC i-motif formation at neutral pH [119]. Moreover, a recent study reports on stabilizing i-motif structure at physiological pH in the solution containing Ficoll and PEG [120].

Proteins

As discussed earlier, the cellular environment has been found to be crowded and has important implications for cellular processes, including protein folding and binding, enzyme kinetics and gene expression. With the advancement in the studies, various theoretical and computational approaches have been discussed to understand the significant impact of crowded environment on protein structure, stability, nature of binding, etc. [121]. For instance, a recent report used a comprehensive study using molecular simulations for protein interaction to characterize diffusion patterns in a crowded milieu. The translational and rotational diffusion rates were determined with a wide range of concentrations of the most abundant protein type in *E. coli* in heterogeneous and self-crowding conditions. The results were found in well agreement with the available data. Such observation proved another benchmark to showcase the approach of structure-based simulation to study the impact of molecular crowding on protein structure and interaction [122]. Also, a very informative article by Das et al. explained how a traditional crowding theory about a balance between a stabilizing entropic effect and stabilizing/destabilizing enthalpic effect failed to explain the experimental observations and thus highlighted the role of associated water dynamics in determining the protein stability under crowded environment. It was found that the associated water molecule plays a vital role in the stability of the protein in the presence of crowding agents (Ficoll-70, dextran-40, PEG-35). Slow water dynamics characterizes its rigid association with a lower degree of freedom and hence causes entropic stabilization and enthalpic destabilization, and the reverse is true for faster water dynamics [123]. Though the interactions stabilizing the three-dimensional structure of proteins are well defined, some gaps still exist because they function in a crowded cellular milieu while the studies are done in simple buffered solutions. In

this part, we explore how crowders impact proteins' shape, stability, and folding. It has been demonstrated that protein complex formation in a crowded environment is crucial to understanding the physiological and pathological nature of cellular life [124,125].

Shape of protein molecules

Multidomain proteins that exist in close and open functional states or proteins going through transitions between compact and elongated forms are among the possible targets that might be affected by the presence of crowders. The external surface lipoprotein of *Borrelia burgdorferi*, known as VlsE, undergoes antigenic variation due to a complex gene conversion mechanism. This lipoprotein is known to be important in the immune response to Lyme disease *Borrelia*. It has an extended football form with floppy loops at every end surrounding a helical center [126]. Using a variety of *in silico* and *in vitro* methods, Homouz et al. studied the impact of molecular crowding on structural changes and the shape of the aspherical protein VlsE. They demonstrated that the presence of urea and high concentration of Ficoll-70 circumstances cause the development of non-native β-structure [127]. An earlier study also reported that from 0 to 400 mg mL^{-1} of Ficoll-70 (in buffer pH 7, 20°C), the helix content in VlsE increases by 52–80%, and when using Ficoll-70 at 250 and 300 mg mL^{-1}, a unique non-native form of VlsE was observed which was said to be β-turn or sheet [128].

A study by Wang et al. included calmodulin (CaM) protein that has two N- and C-lobes and a four helix-loop-helix motif known as EF-hand binding calcium. It was shown that under crowding conditions of Ficoll-70, the structure changed into a more compact form. Literature also reveals that increased quantities of macromolecular crowding agents impact CaM's helicity, conformation, and EF-hand orientation. This suggests that crowding may be a factor in CaM's unrestrained behavior during target selection inside the cells [129]. Similarly, Samiotakis et al. used computer simulation and experimental studies to demonstrate how crowding conditions affected the folding, structure, and activity of phosphoglycerate kinase (PGK). Although the precise change in structure and folding is yet unknown, the report indicates that the two subunits in the PGK structure got closer in a crowded environment driven by Ficoll-70. Macromolecular crowding leads to the generation of more compact, enzymatically active structures of PGK [130]. Conformational changes of Hb from native to non-native form at different concentrations of BSA were also monitored using intrinsic fluorescence and ATR-FTIR spectroscopy [131].

In addition to having an immediate impact on the stability and conformational behavior of proteins, crowding can alter protein function and structure. In the presence of neutral bystander molecules, the compact form of proteins is favorable compared to the unfolded form [132].

Conformational stability of proteins

The stability of folded proteins is strongly influenced by the concentration and surrounding cosolutes. The molecular-level mechanisms of protein stabilization are well understood using ^{19}F nuclear magnetic resonance (NMR), showing that PEG-mediated stabilization is accompanied by significant heat release, and entropy disfavors the folding [133]. Macromolecular crowding by PEGs reduces protein breathing, thus turning local unfolding into global unfolding [134]. It is also known that the presence of dextrans significantly boosted the mechanical stability of ubiquitin. This accounted for an enhancement of the unfolding free-energy barrier computed in the scaled-particle theory [135]. While the secondary structure of cytochrome *c* remained unaltered in the presence of Dextran-70 and Ficoll-70, the T_m increased by 5–10°C. Intriguingly, guanidine hydrochloride, which generally acts as a protein denaturant, enhanced the stabilization effect of crowding agents. Also, small dextran molecules like Dextran-40 had a more significant stabilizing impact on thermal stability (ΔT_m = 10–20°C) as compared to Ficoll-70 and Dextran-70 [136]. Macromolecular crowding influences the stability of Bacillus subtilis Cold shock protein B (BsCspB) by increasing loop rigidity, which contributes to the thermodynamic stabilization of the protein. Crowding agents notably diminish the mobility of non-hydrogen-bonded NH groups with solvent protons, thereby enhancing protein stability [137].

Macromolecular crowding has also been shown to enhance enzyme catalytic activity, either by changing the enzyme's conformation to a higher activity state or by increasing the enzyme's effective concentration due to a decrease in accessible free water [138–140]. For instance, BSA and PEG 6000 were shown to mediate a significant increase in the catalytic activity of glucose-6-phosphate dehydrogenase (caused by crowding-promoted stabilization of active dimer) under conditions promoting its dissociation and inactivation in dilute solutions [140]. Ficoll-70 affects the thermal stability of creatine kinase (CK), reflected in an increase in T_m at circular dichroism melting studies. Both native and denatured states of CK showed a compaction effect in the presence of Ficoll-70 [141]. These experimental results accorded well with the excluded volume theory.

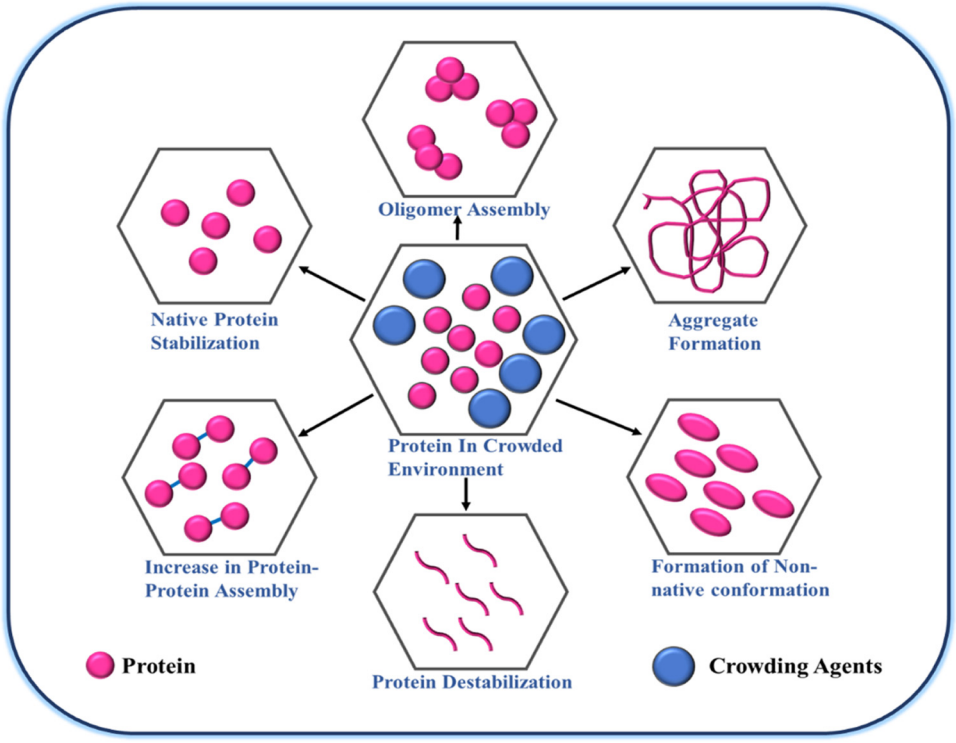

Figure 6: Illustration of protein's responses to macromolecular crowding.

Contrary to beneficial effects, macromolecular crowding may also exert some adverse effects on biological processes. This suggests that high macromolecular concentrations *in vivo* may significantly impair a cell's ability to function normally. Some of the favorable and unfavorable effects of crowding on proteins are depicted in Figure 6. There are substantial shreds of evidence that contradict the conclusions; for example, under crowded situations of PEG-2000 and Dextran-70, the activity of recombinant human brain-type CK (rHBCK) decreases [142]. Further, myoglobin unfolding occurs when Ficoll-70, Dextran-70, or 40 are present [143]. The inclusion of Ficoll-400 as a crowding agent has been shown to promote the denaturation of pepsin and trypsin, impacting their stability and structure when unfolded. The fluctuations observed in the hydrodynamic radius measurements of pepsin and trypsin can be attributed to the influence of excluded volume effects caused by the crowding agents [144]. Also, due to increased protein–protein interactions, the native state stability of chymotrypsin inhibitor 2 decreased in the protein crowders environment. This finding was further supported by molecular simulations and NMR measurements [145].

When translated on polysomes, several unfolded polypeptide chains with accessible hydrophobic residues could be highly susceptible to intermolecular interactions and aggregation [146,147]. Along with these, macromolecular crowding promotes protein aggregation. For α-synuclein, an intrinsically disordered protein implicated in Parkinson's disease, it was found that in the presence of higher molecular weight PEGs or Ficolls, protein aggregation increases due to volume exclusion [148,149]. However, the aggregation is not merely because of the excluded volume effect; it also relies on the co-solute properties, causing changes in hydration factors [150]. Macromolecular crowding using the replication system of bacteriophage T7 has been examined, concluding that it affects several aspects of DNA replication. The DNA helicase activity was increased, whereas the sensitivity of DNA polymerase to salt was reduced. Small-angle X-ray scattering analysis demonstrated that the complex between DNA helicase and DNA polymerase/trx is far more compact in a crowded environment [151]. In their elegant mini-review, Mittal et al. have discussed the deleterious effects of macromolecular crowding on protein structures and stability [152]. In Table 1, we have enlisted a few important examples of macromolecular crowding on different biomacromolecules.

Lipids

Biomacromolecular crowding, though less explored so far, has also been depicted for lipid-rich cellular membranes, where the assorted lipid bilayer and ubiquitous peripheral

Table 1: List of important macromolecular crowding agents with their effect on biomacromolecules

Biomacromolecules	Crowding agents	Effect of crowding agent on biomacromolecules	References
	PEG-8000	T_m of poly(dA)·poly(dT) duplex increased by 5°C	[65]
	PEG-200	T_m of 5′-dATGCGCAT-3′ decreased by 11.8°C	[67]
DNA	PEG-200	T_m of 5′-ATGCGCAT-3′ and 5′-A27GCG-3′/5′-CGCT27-3′ increases by approximately 15 and 10°C, respectively	[68]
	PEGs	T_m of triplexes T_{18}*$(AT)_{20}$, T_{17}I*$(AT)_{20}$, and $T_{16}I_2$*$(AT)_{20}$ increased by 15–16°C	[95]
	PEG (Avg MW = 300)	Alteration the G-quadruplex topology of $d(G_4T_4)_3G_4$ and $d(G_4T_4G_4)_2$ from anti-parallel to parallel conformations	[112]
	Ficoll-70	Development of non-native β-structure for protein VlsE	[128]
	Ficoll-70	Change in helicity, conformation, and EF-hand orientation of CaM protein	[129]
	Ficoll-70	Compaction of protein PGK	[130]
	Ficoll-70 and Dextran-70 in the presence of guanidine HCl	Increase of T_m of cytochrome C by 10–20°C	[136]
Proteins	Ficoll-70	Compaction and increase in T_m of protein CK	[141]
	PEG-2000 and Dextran-70	Decrease in activity of r-HBCK protein	[142]
	Ficoll-70	Unfolding of myoglobin	[143]
	Glycocalyx polymers	Change in morphology of lipid membrane	[153]
	Green fluorescent protein (GFP)	Bending of membrane protein	[153]
	Protein and cholesterol	Enhances cell membrane viscosity	[154]
Lipids	Fluorescent protein (GFP) and nanolipoprotein particles	Facilitate the amalgamation of lipids within the bilayer	[155]
	PEG-8000 and Dextran 500k Green	Reduction of hydrodynamic radius resulting in improved passive encapsulation of biomacromolecules within lipid vesicles	[156]
	Sorbitol	Irreversible compaction in lipid vesicles	[157]

and integral proteins assemble into a complex fluid mosaic structure [158–160]. It is predicted that about one-third of a mammalian cellular proteome is membrane-associated proteins. The extent of crowding mediated by membrane proteins, polysaccharides show a high cell type specificity, for example, proteins of red blood cells occupy 25–30% of the total plasma membrane area, 50% within the light-sensitive membrane of the eye rod, and up to 80% in the densely packed thylakoid membranes [161,162].

Crowding typically influences the diffusive transport of proteins and lipids, alters the conformational dynamics of proteins within lipid bilayers and membrane interfaces, and consequently modulates membrane organization. Although the approaches to study the crowding in living cells and reconstituted systems are currently restricted, attempts have been made to assess the crowding in lipid membranes using non-invasive fluorescence-based approaches. The probing of interfacial crowding in synthetic and native membranes has been well described using a genetically encoded sensory protein [163]. The hydrophobic length (and shape) of proteins have been suggested as a determining factor for the intracellular localization of transmembrane proteins. Various protein species with different geometrical shapes (cylindrical, non-cylindrical, peripheral) that are initially randomly distributed in a membrane may eventually lead to oligomerization and clustering of integral membrane proteins due to membrane-mediated interactions. The conformation and the function of membrane systems may be affected by protein crowding; for example, it may induce deformations of lipid bilayers into curved structures such as buds and tubules (Figure 7). Furthermore, crowding gave rise to an entropic tension in the membrane, affecting conformational transitions related to changes in the projected area and the circumference of a membrane protein [160].

Protein-protein crowding and its prevalence in cellular membrane-shaping processes have been elegantly summarized by Ruhoff et al. Thus, crowding has been shown to generate spontaneous vesicle formation and tubular morphologies on cell and model membranes. This indicates crowding as a significant player involved in the bending of membranes which allows for the uptake of nutrients, waste disposal, cell migration, and much intra- and extracellular communication [153,163]. The heterogeneity of biomembranes indicates that cells could regulate the diffusion-limited reaction rates via varying local viscosities. On these lines, a recent study demonstrated that protein crowding and cholesterol increase cell membrane viscosity in a temperature-dependent manner [154]. The

basic mechanism governing membrane encapsulation of DNA in cells has been investigated using fluorescence microscopy and giant unilamellar vesicles, showing how molecular crowding promotes the spontaneous enveloping of model DNA into lipid bilayer membranes [164]. The cell membrane largely has proteins and polysaccharides inserted and embedded within, creating a highly crowded environment. Macromolecular crowding decreases intracellular signaling, active protein transport, metabolic processes, and cytoplasm fluidity [14]. Furthermore, macromolecular crowding has been shown to slow down the diffusive motion of individual lipids and proteins, hence affecting time scales of encountering, association, and re-binding. Hence, protein diffusion, aggregation, and configurational alterations are expected to be studied in crowded membranes. Such studies offer great promise for gaining an in-depth understanding of cell membranes and organelle membranes and reveal more about the impact of crowding on cell membranes and working cellular mechanisms.

Conclusion and future perspective

Though macromolecular crowding is gaining recognition due to the potential to change our understanding of the cellular environment fundamentally, our current scientific knowledge about macromolecular crowding is still in its formative years. Intracellular biochemical interactions are largely affected by macromolecular crowding, which promotes molecular associations and structural reorganization of the cell. The technological advances have yielded insights into protein behavior in the cellular milieu.

However, these studies have some limitations, like (1) clarification of the role of environmental regulation in cells. The correlation between the peril of diseases and crowding driven by modifications in nucleic acid stability must be quantified. (2) Evaluation of cellular environments involving various cell types and cell cycles as well as the implementation of intracellular studies for nucleic acid stability. (3) Prediction challenges for non-canonical structures, especially G-Quadruplexes, because of the complex interaction between co-solutes and non–canonical structures. It is crucial to evaluate the basic biophysical characteristics, including the tertiary structures of proteins, in various aqueous solutions, and under diverse circumstances. No universal crowding agent is available that accurately replicates intracellular crowding conditions for many biological systems. Due to crowding agents' size, shape, concentration, and surface charge, they do not consistently produce "pure" crowding effects on certain biomacromolecules of interest. Anticipating the impact of a certain co-solute on proteins and nucleic acids collectively is challenging, hence requiring additional experimental and computational investigations considering all the aspects of soft interactions,

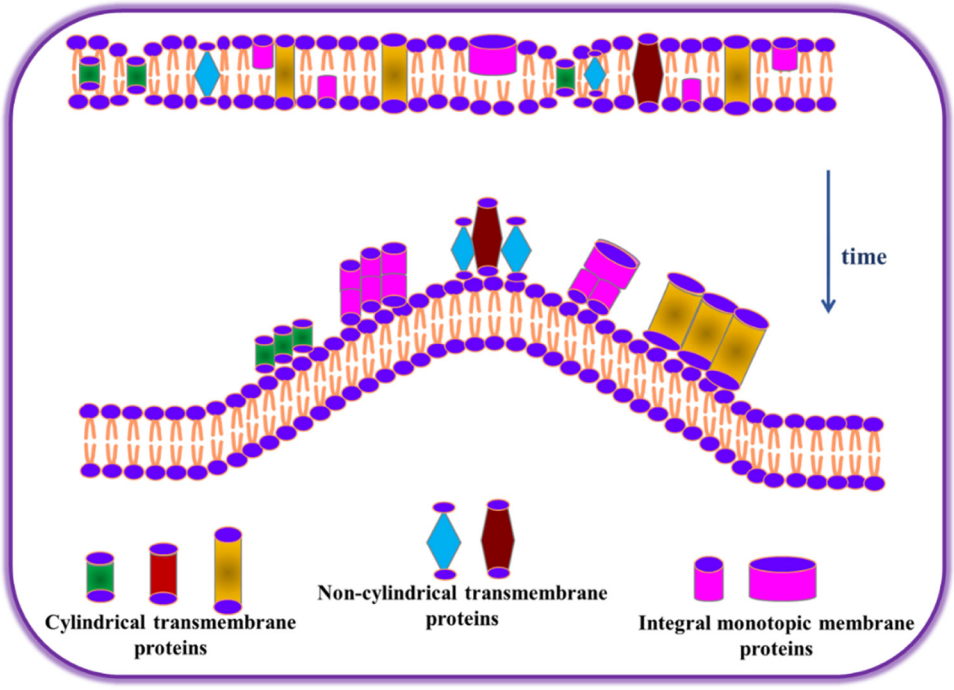

Figure 7: Lipid membrane deformation induced by membrane proteins.

excluding volume and water dynamics. Macromolecular crowding is affected by diverse components, sometimes leading to a decrease in the catalytic activity of enzymes by favoring protein aggregation, forming non-native conformations, reducing the diffusional mobility of reactants, and diminishing the hydration layer.

The evidence demonstrates that protein behavior in isolated solutions diverges from the physiological environment. Therefore, predicting the structure and stability of these biomacromolecules is challenging due to the spatiotemporal variations in cellular circumstances. A network of interactions describing fundamental cellular processes could be best understood by applying experimental and theoretical modeling studies. Many aspects remain largely unexplored, especially the incomplete information about the nature of macromolecular crowding effects. There is still limited evidence showing that the source of crowding effects resides in the macromolecular nature of the crowding molecule because the controls comparing the monomer to its macromolecular counterpart are rarely performed. Although several modeling and simulation studies consider chemical interactions, experimentally derived information is limited. These limitations need to be addressed. Further, investigations are required to find universal parameters that should describe interactions, crowding, cell cycle, location, and cation to demonstrate the stability of nucleic acid structures. Understanding these dynamics is crucial for personalizing gene expression patterns and creating personalized treatment since cellular conditions differ between individuals. To fully understand the exact status of the biomacromolecules in the cellular environment, the precise mechanism of interaction of nucleic acids and proteins with different solvents, such as water-soluble co-solutes, needs to be elucidated.

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