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# Influence of Osmolytes and Ionic liquids on the Bacteriorhodposin structure in the absence and presence of oxidative stress: A combined experimental and computational study

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**Abstract**: Understanding the folding and stability of membrane proteins is of great importance in protein science. Recently, osmolytes and ionic liquids (ILs) are increasingly being used as drug delivery systems in the biopharmaceutical industry. However, the stability of membrane proteins in the presence of osmolytes and ILs is not yet fully understood. Besides, the effect of oxidative stress on membrane proteins with osmolytes or ILs has not been investigated. Therefore, we studied the influence of osmolytes and ILs as co-solvents on the stability of a model membrane protein (i.e., Bacteriorhodopsin in purple membrane of *Halobacterium salinarum*), using UV-Vis spectroscopy and molecular dynamics (MD) simulations. The MD simulations allowed us to determine the flexibility and solvent accessible surface area (SASA) of Bacteriorhodopsin protein in the presence and/or absence of co-solvents, as well as to carry out principal component analysis (PCA) to identify the most important movements in this protein. In addition, by means of UV-Vis spectroscopy we studied the effect of oxidative stress generated by cold atmospheric plasma on the stability of Bacteriorhodopsin in the presence and/or absence of co-solvents. This study is important for a better understanding of the stability of proteins in the presence of oxidative stress.

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## Introduction

In recent years, membrane proteins are gaining interest as targets for cellular drugs, and approximately 27% of all human proteins are estimated to be α-helical membrane proteins [1]. Bacteriorhodopsin is a model α-helical membrane protein that acts as a light-driven proton pump present in the plasma membrane of *Halobacterium salinarum* [2]. The bacteriorhodopsin membrane protein structure was extensively investigated using various experimental and theoretical methods. The structure of Bacteriorhodopsin was first revealed by electron diffraction [3,4]. Bacteriorhodopsin consists of 7 transmembrane helices (A–G) surrounding the retinal molecule. When a photon is absorbed by Bacteriorhodopsin, the cycle of the photochemical reaction begins and ends with the pigment returned to its original form[5]. The proton pathway consists of charged residues, such as Glu204, Glu194, Asp212, Asp96, Asp85, and Arg82 [6]. Recently, detailed information on the structure of Bacteriorhodopsin with a resolution higher than 1.3 Å became available [6]. Despite this fact, little information is known about the folding and unfolding parameters of α-helical membrane proteins in the presence and absence of co-solvents.

Protein folding can be manipulated by co-solvents, such as salts, sugars, polyols, amino acids, amine and ionic liquids (ILs) [7–9]. Many preferentially excluded co-solvents can stabilize proteins, reduce their solubility and aid in the crystallization. Some of the co-solvents are used by the organisms living in salty environment; they increase the osmotic pressure of the cytoplasm to withstand the salty environment [10]. Some co-solvents, for instance sugars and polyols, play a crucial role in the development of pharmaceutical protein preparations [10]. A large concentration of inorganic salts can disturb the protein functions, and therefore, certain cells use organic salts, for example osmolytes, because they do not interrupt the cell functions and protect the protein structure from inorganic salts. Naturally occurring osmolytes can stabilize the protein and alter the folding equilibrium away from the degradation or aggregation of proteins [11]. Another class of protein stabilizer are synthesized co-solvents, such as ionic liquids (ILs). ILs are called "designer solvents" because of the huge number of possible combinations of cations and anions [12]. The physicochemical properties of ILs include low toxicity, high chemical/thermal stability, controllable hydrophobicity/hydrophilicity and negligible vapor pressure [13–15]. Numerous applications of ILs were reported, where they were used as a solvent for organic synthesis, solar cells, electrochemical studies, extraction/separation/stability of proteins, etc.[16–20]. The efficacy of biocompatible ILs has

been improved in recent years, which resulted in the implementation of handling and storage activities towards biomolecules, in comparison to a conventional aqueous medium.

In the last few years, osmolytes and ILs are being used in biopharmaceutical formulations for drug delivery [21–23]. During the manufacturing process and administration of the drug, protein formulations require more care, as any variations in the physical parameters can result in inactivation, denaturation, and protein aggregation. Osmolytes and ILs are increasingly being used as co-solvents to overcome these problems, but limited information is available on the exact role of osmolytes and IL in determining the protein stability, and on the effect of oxidative stress on protein folding in the presence of osmolytes or ILs. Therefore, in this study, we examined the stability of Bacteriorhodopsin in the presence of osmolytes (namely, sorbitol and trehalose) and ILs (namely, tetrabutylammonium nitrate (TBAN), tetrabutylammonium nonafluorobutanesulfonate (TBFS) and tetrabutylammonium methanesulfonate (TBMS)) using UV-Vis spectroscopy and molecular dynamics (MD) simulations.

UV-Vis spectroscopy is frequently used by researchers to study the Bacteriorhodopsin behavior against thermal, detergents and light-induced denaturations [24–28]. Moreover, UV-Vis spectroscopy was used to investigate the acid denaturation on the photoactive yellow protein from *Ectothiorhodospira halophila*, a member of water-soluble photoreceptors family known as Xanthopsins [29]. MD simulations were used previously to study the effect of sodium dodecyl sulfate (SDS) micelles on the Bacteriorhodopsin structure [30], and also to examine the structural aspects of other membrane proteins, as reported in the review by Almeida *et al*. [31]. UV-Vis spectroscopy is often used to evaluate the role of osmolytes and ILs on various proteins, such as myoglobin, hemoglobin, mushroom tyrosinase, cytochrome c, bovine serum albumin, catalase, stem bromelain, etc [32–40]. Similarly, MD simulations have become a popular tool to assess the effect of osmolytes and ILs on numerous proteins [41–46]. Denaturation of Bacteriorhodopsin can be predicted using UV-Vis spectroscopy [24,26]. This technique is cheap and easy to handle compared to other complex and costly techniques, such as X-ray crystallography, NMR, Cryogenic electron microscopy (cryo-EM), etc. Moreover, sample preparation and unfolding measurements of Bacteriorhodopsin with and without osmolytes or ILs can be easily done using UV-Vis spectroscopy. However, UV-Vis spectroscopy cannot provide information about the fluctuations in residues of Bacteriorhodopsin in the presence and absence of co-solvents. This can be overcome by MD simulations, which provide more details about the conformation of Bacteriorhodopsin. Hence, the combination of UV-Vis spectroscopy and MD simulations can help to understand the stability of Bacteriorhodopsin in the presence and absence of co-solvents.

Further, we treated the Bacteriorhodopsin with cold atmospheric plasma (CAP), i.e., a new treatment modality to create oxidative stress, in the presence and absence of co-solvents, to find out the effect of oxidative stress. Few studies already reported the effect of CAP on different proteins, namely horseradish peroxidase [47], lysozyme [48], myoglobin [49], lipase [50], hemoglobin [19], α-chymotrypsin [51], MTH1180 [52] and cytoglobin [53]. However, the effect of CAP on membrane proteins is still unknown in literature to the best of our knowledge.

**Materials and Methods**

**Materials.** The Bacteriorhodopsin protein, sorbitol, trehalose, TBAN, TBFS and TBMS were supplied by Aldrich Chemical Co. (USA). All chemicals and reagents were used without further purification. The concentrations of $H_2O_2$, $NO_2^-$, and $NO_3^-$ were measured by methods provided in our previous work[54,55].

**Dielectric barrier discharge**. Experiments were carried out using a scalable DBD device, as described in previous work[56]. The device was set in ambient air at atmospheric pressure. DBD plasma was generated between the electrodes by supplying a 10 kHz AC high voltage. The peak-to-peak discharge voltage and current were 9.2 kV and 0.2 A, respectively. The corresponding discharge power density was 1.49 W/cm$^2$, which was deduced from a voltage/charge Lissajous plot.

**UV-vis spectra.** UV absorbance was measured with and without osmolytes or ILs using a plate reader in 24 well plates. A 0.5 mg/ml concentration of sample was used for the measurements, with each spectrum being the average of six spectra.

**Reactive species in buffer solution**: We analysed the concentrations of $H_2O_2$, $NO_2^-$, and $NO_3^-$ after the DBD plasma treatment. The concentrations of $H_2O_2$, $NO_2^-$ and $NO_3^-$ were approximately 80, 320 and 486 μM, respectively, for 20 min DBD treatments in buffer solution.

**pH measurement.** After the buffer solution was exposed for 20 min to the plasma, its pH was measured using Horiba scientific instruments. All measurements were carried out in triplicate. No variation in pH of the solution was observed after plasma treatment.

**Sample preparation.** The protein was added to 2 ml screw-capped vials in 10 mM Tris HCl buffer (pH 7.2), kept on ice for 2 h to attain complete equilibrium after blending the solution. Similarly, the protein was dissolved in a mixture of 500 mM of osmolytes (sorbitol or trehalose) and ILs in Tris-HCl buffer and the solution was kept for 2 h to attain complete equilibrium after blending the solution. The samples were treated at 3 mm distance from the plasma exposed for 20 min, at a humidity of 40-50% RH, and were then incubated for 4 h in ice after plasma treatment. Six samples were treated for each condition to minimize the error.

**Molecular dynamics simulations**. The structure of Bacteriorhodopsin was obtained from the (RCSB) protein data bank ( PDB ID: 5ZIL)[6]. The MD simulations were performed with the GROMACS 5.1.2 package [57] using the CHARMM27 all atoms force field [58]. The protein was solvated with water, described by the simple point charge (SPC) explicit solvent model [59]. Subsequently, to neutralize the system, $Na^+$ or $Cl^-$ ions were added randomly to the simulation box by replacing the water molecules. The system was energy minimized with the steepest descent method. Furthermore, applying a position restraint on the heavy atoms of the system, the equilibration was performed at 300 K and 1 bar in the NPT ensemble for 100 ns, employing modified Berendsen thermostat [60] and Parrinello-Rahman barostat [60,61], respectively. Subsequently, the MD simulations were performed for 100 ns, again applying the NPT ensemble at 300 K and 1 bar, now without position restraints. In all simulations a time step of 2 fs was used. Finally, we calculated the root mean square deviation (RMSD) of Cα atoms, root mean square fluctuations (RMSF) of protein residues, radius of gyration (Rg), principle component analysis (PCA) and solvent accessible surface area (SASA) values, to check the stability, flexibility and surface area of the protein that is accessible to the solvent. Besides simulating the Bacteriorhodopsin in water, we also performed the same simulations by adding 500 mM of sorbitol, trehalose, TBAN, TBFS and TBMS to the water. The CHARMM force field parameters for osmolytes (i.e., sorbitol and trehalose) and ILs (i.e., TBAN, TBFS and TBMS) were obtained using SWISSPARAM server [62]. Analysis of the MD results, as well as calculation of the average values, was performed using the last 20 ns of the MD trajectory.

## Results and discussion

In our previous study, we observed that osmolytes and ILs can protect the structure of various proteins, such as Myoglobin, Hemoglobin, α-Chymotrypsin and Succinylated Con A[17,63–65]. Recently, we reported that 500 mM of trehalose and sorbitol can protect the Myoglobin structure against CAP and gamma rays [49]. Thus, we used 500 mM of osmolytes and ILs to explore the stability of Bacteriorhodopsin in purple membrane against CAP treatment.

### Stability of Bacteriorhodopsin membrane protein in the presence and absence of osmolytes

Figure 1 illustrates the UV-vis spectra of the Bacteriorhodopsin in buffer (control), sorbitol and trehalose. The absorbance at 560 nm is due to the π-π* transition of the retinal chromophore of Bacteriorhodopsin protein [26,28]. Moreover, another minor absorption band from 450 to

320 nm is due to transitions of higher excited states of the chromophore [66]. The peak at 560 nm serves as a convenient spectroscopic probe to monitor the unfolding of the Bacteriorhodopsin protein [24,26,28]. Thus, it is useful to investigate the peak at 560 nm to study the folding and unfolding properties of the Bacteriorhodopsin protein in the presence of different co-solvents. The strong absorption band at 560 nm and a minor absorption band at 380 nm were observed for Bacteriorhodopsin in buffer (see Figure 1). Free retinal peak is observed at 380 nm for Bacteriorhodopsin in purple membrane [27]. With the addition of sorbitol, an increasing intensity was observed at 560 nm in comparison with the control, as shown in Figure 1a. On the other hand, addition of trehalose resulted in quenching of the peak at 560 nm (see Figure 1b). However, the peak intensity from 450 to 320 nm was not altered with the addition of osmolytes in comparison to the control measurement. The absorbance intensity decreased with the addition of trehalose, which may be due to the reduced exposure of retinal chromophore to the solvent. On the other hand, the increase of absorbance with the addition of sorbitol may be due to the slightly higher exposure of chromophore to the solvent. The absorption spectra in the presence of sorbitol and trehalose at 560 nm are similar to control samples, i.e., no peak shift was observed with addition of these co-solvents. Although the peak intensity at 560 nm was increased or decreased for sorbitol and trehalose, respectively, this suggests that both sorbitol and trehalose can influence the Bacteriorhodopsin bound retinal differently. The free retinal peak at 380 nm was not influenced by addition of both co-solvents, which implies that the addition of co-solvent does not denature the Bacteriorhodopsin structure. The decrease in peak at 560 nm with blue shift was observed in previous research during the thermal denaturation of Bacteriorhodopsin, where at the same time the band intensity at 380 nm increased, indicating the formation of free retinal [27]. Hence, we conclude that the addition of osmolytes does not denature the Bacteriorhodopsin structure, and the structure of the protein becomes more compact with addition of trehalose compared to sorbitol. A similar change in absorbance intensity was also observed for other protein systems with addition of sorbitol or trehalose [40,67].

To understand the conformational changes occurring in Bacteriorhodopsin protein structure, in the presence of osmolytes, we performed MD simulations. Specifically, we calculated the RMSD, RMSF, Rg, PCA and SASA, to evaluate the stability of Bacteriorhodopsin protein in the presence of sorbitol and trehalose. The RMSD provides evidence of the flexibility of the protein with and without co-solvents. Figure 2a shows the RMSD of Cα atoms of Bacteriorhodopsin protein in water and water-containing osmolytes. The calculated average RMSD values were found to be $0.18 \pm 0.06$ nm, $0.24 \pm 0.03$ nm and $0.16 \pm 0.40$ nm in the

presence of water, water+sorbitol and water+trehalose, respectively. This indicates that the flexibility of Bacteriorhodopsin increased with the addition of the sorbitol, whereas it stabilizes more in the presence of trehalose. Figure 2b illustrates the RMSF of Bacteriorhodopsin in water and water-containing osmolytes. The average RMSF of Bacteriorhodopsin in water was $0.11 \pm 0.08$ nm, whereas in sorbitol and trehalose its values were $0.12 \pm 0.33$ nm and $0.09 \pm 0.10$ nm, respectively. The residues between 30-43, 157-166 and 197-203 faced higher fluctuations in the presence of sorbitol compared to the control and trehalose. This again shows that the flexibility of Bacteriorhodopsin increased in the presence of sorbitol, which is not the case for trehalose (i.e., more or less the same as in the case of the control). We also calculated the Rg for Bacteriorhodopsin in the presence or absence of osmolytes (see Figure 2c). Rg is an indicator of the protein structure compactness. The average Rg values for Bacteriorhodopsin in water, water+sorbitol and water+trehalose were $1.86 \pm 0.05$ nm, $1.90 \pm 0.07$ nm and $1.84 \pm 0.06$ nm, respectively. These results indicate that the Bacteriorhodopsin structure in the presence of trehalose was found to be more stable and compact than in the presence of water and water+sorbitol. The RMSD, RMSF and Rg values were higher for Bacteriorhodopsin in water+sorbitol, which supports our experimental data, where the stability of Bacteriorhodopsin decreased in the presence of sorbitol. To further support the RMSD, RMSF and Rg, we calculated SASA and carried out the PCA analysis for the protein in the absence or presence of osmolytes. The PCA results provide an information about the total phase space that each protein can occupy. In the PCA, collective motion of the Cα is studied by plotting the projection of the first eigenvector (representing the direction of the first highest motion) versus the projection of the second eigenvector (representing the second highest motion). The Cα-atomic positional fluctuations were found to be 2.43, 2.52 and 1.57 $nm^2$ for the water (control), water+sorbitol, and water+trehalose, respectively (cf. Figure 3a). This also revealed that the structure of Bacteriorhodopsin in the presence of trehalose was more compact than in water and water+sorbitol. Figure 3b shows the SASA results, which give information about the surface area of the protein that is accessible to the solvent. The average SASA values of Bacteriorhodopsin in water, water+sorbitol and water+trehalose were $111.5 \pm 0.8$, $126.7 \pm 0.3$ and $110.5 \pm 0.5$ $nm^2$, respectively. This indicates that Bacteriorhodopsin in water+trehalose has lowest accessibility to the solvent, whereas, it is higher in water+sorbitol. Our RMSD, RMSF, Rg, SASA and PCA analysis results revealed that the addition of sorbitol increases the flexibility and surface of Bacteriorhodopsin accessible to the solvent, while trehalose results in a decrease of the flexibility and less surface area of the protein becomes accessible to the solvent compared to the control sample (i.e., without co-solvent). Hence, this result support our

above mentioned theory that the addition of trehalose leads to an increase of the Bacteriorhodopsin compactness.

In the previous work, it was reported by single molecule force spectroscopy that betaine, taurine and ectoine osmolytes can stabilize the Bacteriorhodopsin [68]. The authors also concluded that these osmolytes can act as a stabilizing agent against the denaturation stress for membrane protein. In another work, the authors observed that trehalose acts as better stabilizer for α-amylase as compared to sorbitol, sucrose and glycerol [69]. Similarly, during spray drying, trehalose stabilizes the glutamate dehydrogenase enzyme better than sorbitol [70]. On the other hand, other authors observed that both sorbitol and trehalose equally stabilise the myoglobin structure [35]. Hence, the stability of the protein varies with osmolytes, although in our current study trehalose stabilizes the Bacteriorhodopsin better than sorbitol

**Stability of Bacteriorhodopsin membrane protein in the presence and absence of ILs**

Figure 4 presents the UV-vis spectra of Bacteriorhodopsin membrane protein in TBAN, TBFS and TBMS ILs. Like in the buffer (control), the broad absorption peak at 560 nm and a minor absorption band at 380 nm were observed for all ILs. The quenching at 560 nm was observed with the addition of TBAN and TBFS ILs, compared to the control. On the other hand, no quenching was observed in the case of TBMS IL. The decrease in the absorbance intensity for the TBAN and TBFS is a result of a decrease in π-π* transition of retinal chromophore, which may be due to reduced exposure of chromophore to solvent containing ILs. On the other hand, no change in peak intensity at 560 nm for TBMS IL compared to the control was observed, which suggested that TBMS IL stabilizes the protein, similar to the buffer. We assume that the TBAN and TBFS interact with the retinal chromophores, thereby decreasing the π-π* transition, or the presence of these ILs probably decreases the chromophores' exposure to solvent. The TBMS, on the other hand, does not interact with retinal chromophores, therefore no quenching was observed. Additionally, the free retinal peak at 380 nm was not significantly influenced with addition of ILs, which suggests that ILs do not denature the Bacteriorhodopsin structure. On the other hand, quenching of the peak at 560 nm was observed with addition of TBAN and TBFS ILs, this was not the case for the TBMS. This indicates that the addition of TBAN and TBFS may result in an increase of Bacteriorhodopsin compactness, whereas TBMS stabilizes the Bacteriorhodopsin without any interference.

To support these experimental results, we carried out MD simulations to study the Bacteriorhodopsin protein conformational changes that occur with the addition of TBAN, TBFS and TBMS ILs. We again calculated the RMSD, RMSF, Rg, PCA and SASA to evaluate the stability of Bacteriorhodopsin protein in the presence of ILs. Figure 5a shows the RMSD

of Cα atoms of Bacteriorhodopsin in water (control) and in the presence of TBAN, TBFS and TBMS. The calculated average RMSD values for TBAN, TBFS and TBMS were $0.20 \pm 0.07$, $0.19 \pm 0.04$ and $0.17 \pm 0.01$ nm, respectively. Figure 5b illustrates the change in RMSF of Bacteriorhodopsin in the presence of ILs. The average RMSF for Bacteriorhodopsin in TBAN, TBFS and TBMS were $0.11 \pm 0.09$, $0.11 \pm 0.05$ and $0.11 \pm 0.02$ nm, respectively. The higher fluctuation was observed for TBAN at residues between 155-178 compared to other ILs and the control. Figure 5c shows the time evolution of the Rg values. The average Rg values for Bacteriorhodopsin in the presence of TBAN, TBFS and TBMS were $1.86 \pm 0.05$ nm, $1.86 \pm 0.01$ nm and $1.85 \pm 0.06$ nm, respectively. All these results indicate that Bacteriorhodopsin in TBMS was more stable and compact than in the presence of TBFS and TBAN. The RMSD and Rg values for TBAN were slightly higher than for TBFS and TBMS, while the RMSF had no significant difference, except that fluctuations occurred in the presence of TBAN between the 155-178 residues. Thus, the stability order of Bacteriorhodopsin structure in ILs was TBMS > TBFS > TBAN.

Figure 6 shows the PCA and SASA results for Bacteriorhodopsin in water (control) and water with the presence of different ILs (i.e., TBAN, TBFS and TBMS). The Cα-atomic positional fluctuations were found to be 2.23, 2.20 and 2.13 $nm^2$ for TBAN, TBFS and TBMS, respectively (cf. Figure 6a). The PCA results revealed that Bacteriorhodopsin in the presence of TBMS was more compact than in the presence of TBAN and TBFS. The average SASA values of Bacteriorhodopsin in TBAN, TBFS, and TBMS were $116.5 \pm 0.8$, $116.0 \pm 0.8$ and $114.2 \pm 0.1$ $nm^2$, respectively (see Figure 6b). The SASA results revealed that Bacteriorhodopsin in TBAN and TBFS have similar accessibility to solvent, whereas it was lower in TBMS. The RMSD, PCA and SASA results show that there is a decrease in flexibility and accessibility of the Bacteriorhodopsin to solvent, when the TBMS was added compared to TBAN and TBFS. These results also indicate that in general ILs play role in stabilizing the protein structure, although a more thorough study is required for a clear conclusion.

To our knowledge, nobody has previously reported on the stability of Bacteriorhodopsin using ILs, but ILs are used to purify Bacteriorhodopsin from purple membrane of *Halobacterium salinarum* [71]. Additionally, the ILs used in the current work have not been used previously to protect the protein structure. Therefore, this is the first time we provide the application of these ILs in protein folding.

**Effect of cold atmospheric plasma (CAP) on the structure of Bacteriorhodopsin in the absence and presence of osmolytes and ILs**

To understand the effect of CAP on the denaturation of the protein, we focussed on the peak at 560 nm. We observed that after 20 min of plasma treatment of Bacteriorhodopsin protein solution in the buffer, the peak at 560 nm completely disappeared, as shown in Figure S1a. This indicates that the Bacteriorhodopsin protein denatured after plasma treatment. Further, we evaluated the stability of plasma-treated Bacteriorhodopsin protein in the presence of osmolytes (i.e., sorbitol and trehalose). Figures S1b and 7c show that the peaks disappeared again even in the presence of sorbitol and trehalose after CAP treatment. A similar effect was also observed for the Bacteriorhodopsin protein in the presence of ILs, i.e., the peaks at 560 nm disappeared after the treatment with CAP (see Figure S2). Thus, none of the ILs and osmolytes investigated in this study can protect the protein structure against the denaturation effect induced by CAP. Similarly, during the thermal and light induced denaturation of Bacteriorhodopsin as well as its denaturation by sodium dodecyl sulfate (SDS) micelles, the peak at 560 nm disappears [24–28,72]. Thus, it is a convenient spectroscopic probe to monitor the unfolding of Bacteriorhodopsin. These results thus showed the opposite trends compared to the results of our previous studies, where osmolytes and ILs protected the Myoglobin structure against CAP treatment[49,73]. Hence, we can conclude that the protective action of osmolytes and ILs can vary depending on the protein investigated.

The preservation of the drug is critical during manufacturing, transportation and storage, as well as in the course of administration to the patient, because any variations in these factors might result in inactivation, denaturation, and/or protein aggregation [22]. Hence, the biologic formulations have a direct effect on drug stability. As a result, the use of osmolytes or ILs in biopharmaceutical formulations is increasingly being used to overcome these drawbacks. A review by Wlodarczyk *et al.*, mentioned that among all studied biological formulations approved by the FDA, 76.7% formulations contain osmolytes [22]. This is because osmolytes not only improve the formulations but also provide efficient and safe medication to patient [74]. Additionally, osmolytes are less expensive, so that their usage does not significantly influence the cost of biopharmaceutical formulations. On the other hand, the use of ILs in biopharmaceutical formulations is only in the beginning stage. However, a review by Marrucho *et al.*, reveals the use of ILs to solve critical pharmaceutical problems [21] such as low solubility of pharmaceutical compounds and the presence of polymorphs, that affect the efficacy of drugs. Additionally, in the future, ILs can be used as pharmaceutical active compounds given to patients, which can open a wide avenue in the drug market. However, more research is required for understanding the effect of osmolytes and ILs on membrane proteins, before they can be developed as effective drug or drug delivery systems. The role of

membrane proteins is significant in living organism, e.g., in human 50 % of drugs target the membrane proteins. However, the 3D structure of these proteins is difficult to determine by NMR, X-ray crystallography and cryo-EM, being expensive and time consuming. Moreover, the membrane proteins are difficult to analyze in their native environment, due to the presence of lipid membrane [31]. Because of these technical difficulties, little information is available on the membrane proteins. In this respect, computational methods for prediction of the structure of membrane proteins are increasingly gaining attention nowadays [31]. Still, structural prediction of membrane proteins and their interfaces is in an early stage of development. Hence, it is important to study the effect of osmolytes and ILs on various protein and biological systems, the structures of which are available by experimental and computational methods. Additionally, the effect of osmolytes and ILs in the presence and absence of different stress conditions is also important, which helps to minimize the side effects of future drugs and reduces their costs. From this perspective, our current study is interesting and important, as it shows the effect of osmolytes and ILs on the Bacteriorhodopsin in the presence and absence of oxidative stress.

**Conclusions**

The purpose of this study was to evaluate the role of osmolytes and ILs on the stability of membrane proteins, more specifically Bacteriorhodopsin. In addition, we examined the effect of plasma on this membrane protein. Based on both experiments and computer simulations, we conclude that trehalose stabilizes the membrane protein structure better than sorbitol, and TBMS stabilizes the protein structure better than TBFS and TBAN. This may be because both trehalose and TBMS are preferentially excluded from the protein surface. All studied ILs have the same cations but different anions. Thus, we can conclude that anions play an important role in stabilizing the structure of Bacteriorhodopsin. Among trehalose and TBMS, trehalose was a better stabilizer for the Bacteriorhodopsin protein, although quenching at 560 nm was observed for trehalose, the RMSD value of trehalose is lower than TBMS. Additionally, the flexibility of residues was lower for the protein in the presence of trehalose compared to TBMS. This indicates that the overall structure was stable and compact in the presence of trehalose, while higher fluctuations were observed in the presence of TBMS. Finally, we also found that CAP treatment destroys the Bacteriorhodopsin protein structure and that none of the studied osmolytes and ILs can protect Bacteriorhodopsin against this CAP treatment. This study suggests that it is important to check the effect of oxidative stress on the proteins in order to have an effective drug delivery system.

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**Figure Captions**:

**Figure 1:** UV-Vis spectra of the Bacteriorhodopsin protein in buffer (control) and in the presence of the osmolytes (a) sorbitol and (b) trehalose.

**Figure 2**: MD simulation results of Bacteriorhodopsin protein in water (control) and water with sorbitol and trehalose. (**a**) RMSD plots, (**b**) RMSF plots and (**c**) Rg plots.

**Figure 3:** (**a**) PCA and (**b**) SASA results for water (control) and water with sorbitol and trehalose.

**Figure 4:** UV-Vis spectra of the Bacteriorhodopsin protein in (a) tetrabutylammonium nitrate (TBAN); (b) tetrabutylammonium nonafluorobutanesulfonate (TBFS) and (c) and tetrabutylammonium methanesulfonate (TBMS), and comparison with buffer (control).

**Figure 5:** MD simulation results of Bacteriorhodopsin protein in water and water with TBAN, TBFS and TBMS. (**a**) RMSD plots, (**b**) RMSF plots and (**c**) Rg plots.

**Figure 6:** (**a**) PCA and (**b**) SASA results for water (control) and water with TBAN, TBFS and TBMS.

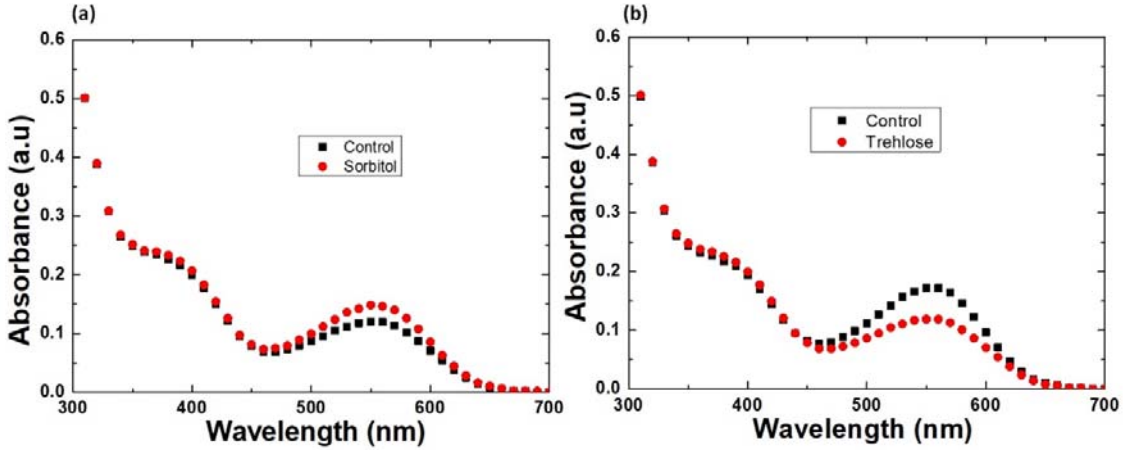


**Figure 1**

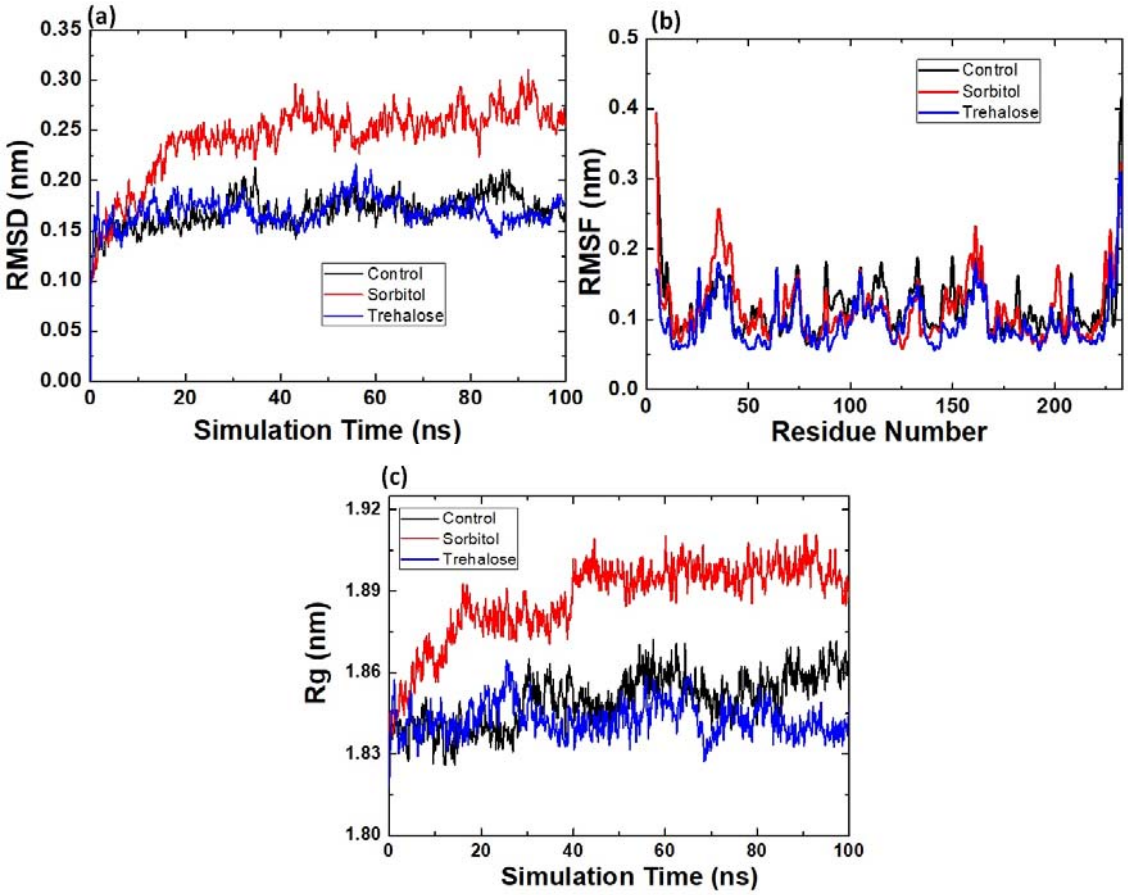


**Figure 2**

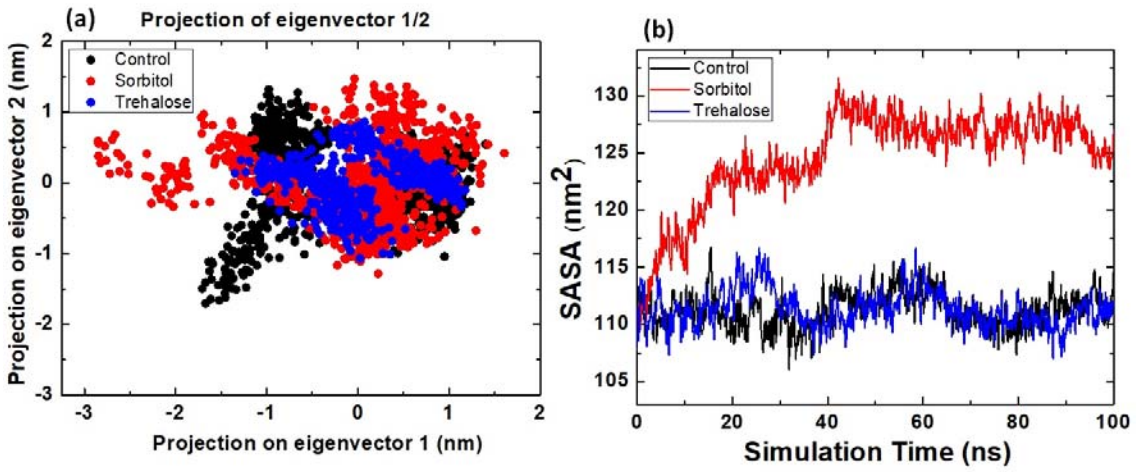


**Figure 3**

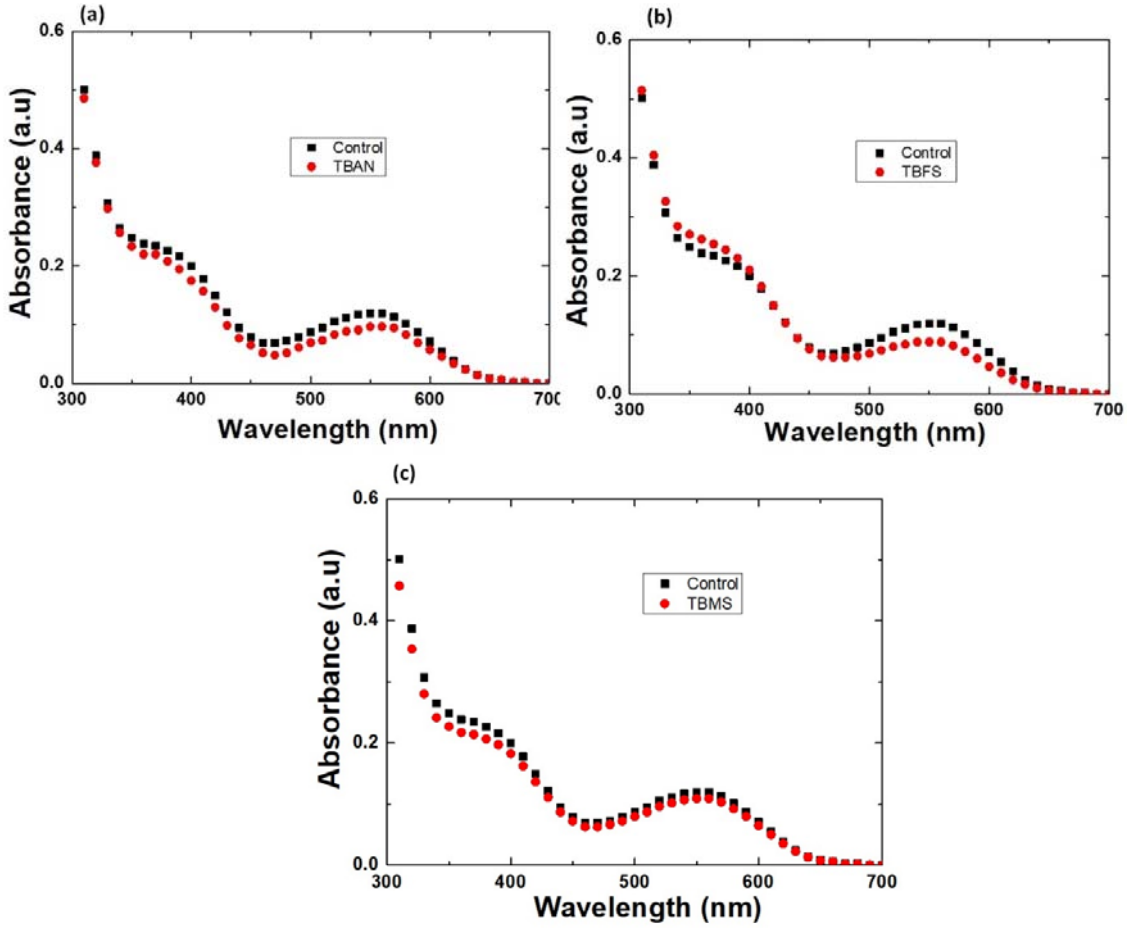


**Figure 4**

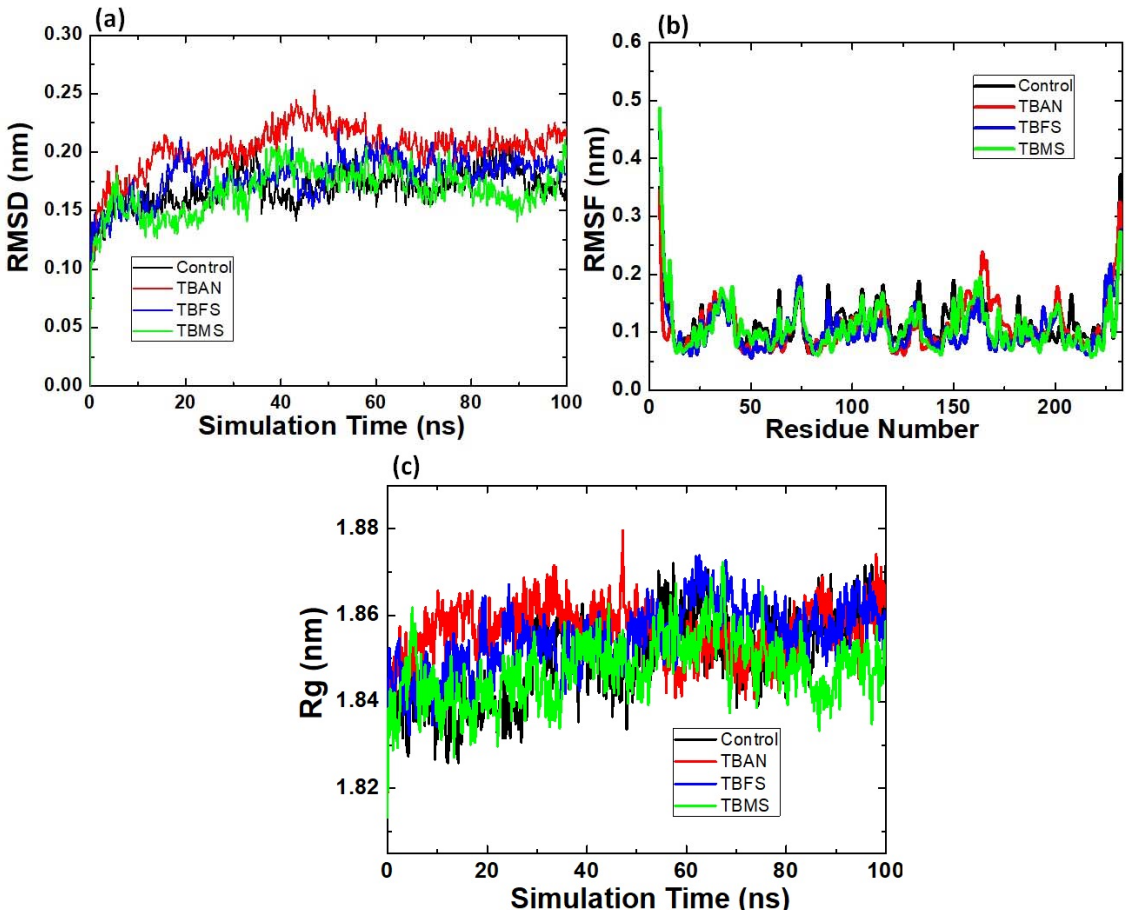


**Figure 5**

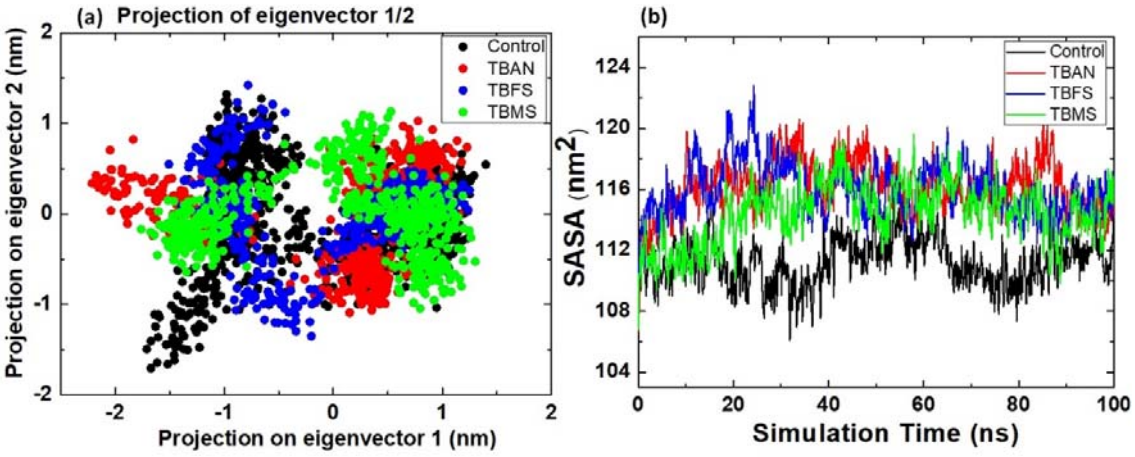


**Figure 6**