Low Temperature Physics/Fizika Nizkikh Temperatur, 2022, Vol. 48, No. 9, pp. 831–837

The mechanism of influence of polyvinyl alcohol (9 kDa) on the formation of ice crystals in aqueous solutions

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Received November 17, 2021, revised April 4, 2022, published online July 18, 2022

Preventing crystallization of the liquid phase during freeze-thawing of cells is one of the main problems that need to be solved for the successful preservation of biomaterial at low temperatures. One highly effective recrystallization inhibitor is polyvinyl alcohol (PVA). However, the mechanisms of its cryoprotective effect have not been finally elucidated. In particular, it is not clear which structural features contribute to the realization of the antirecrystallization properties of PVA in the region of its cryoprotective concentrations. The influence of PVA on solvent crystallization and structural rearrangements of associations of PVA molecules in phosphate-buffered saline (PBS) was experimentally investigated. Solutions of PVA (molecular weight 9 kDa) in PBS were studied by cryomicroscopy and fluorescence spectroscopy methods. It was shown that molecular associations of PVA in PBS undergo a rearrangement of about 0.5–1 wt%, which is accompanied by a change in their size and hydrophilic-hydrophobic properties. PVA also changes the morphological structure of ice upon cooling and prevents crystallization upon heating. It is suggested that the mechanism of the antirecrystallization activity of PVA may be due to the formation of its complexes with the surface of ice crystals.

Keywords: polyvinyl alcohol, crystallization, structural rearrangements, fluorescence.

One of the main factors of cell damage during cryopreservation is uncontrolled growth of ice crystals during the thawing course, such as recrystallization [1, 2]. Therefore, it is promising to develop new cryoprotectors capable of effectively inhibiting ice recrystallization, which along with traditional ice recrystallization inhibitors (IRI) use synthetic compounds, which, like natural antifreeze proteins (AF(G)Ps), have inherent antirecrystallization activity [3–6]. The advantages of synthetic analogues include greater availability, lesser cytotoxicity and lesser immunogenicity compared to natural AF(G)Ps [7, 8]. Among such compounds, polyvinyl alcohol (PVA) deserves attention. In micromolar concentrations PVA exhibits the properties of an effective ice recrystallization inhibitor and slows down the freezing efficiency of water in some cells, including erythrocytes [3, 8–10]. However, these results are preliminary and require additional research. In particular, it is important to clarify the role of the structure and supramolecular organization of polyols in solution in understanding the mechanisms of their suppression of recrystallization processes during freezing and thawing.

Recent studies have established that carbohydrate-based surfactants are inhibitors of ice crystallization below the critical concentration of micelle formation (CCM) [11, 12]. At the same time, analysis of several AF(G)Ps showed that all of them have hydrophobic domains in their structure. So, a possibility exists, an inhibitor adsorbed on the surface of ice nanocrystals can hamper their growth and make an additional contribution to the manifestation of IRI activity. This idea is in line with the results [11], which showed that the formation of micelles in the solutions of nonionic surfactants is not necessary for the manifestation of IRI activity. There was also not found no connection between the ability of glycans to form hydrogels and their IRI activity. At the same time, the level of hydration of carbohydrates is important for the detection of IRI activity.

A large number of carbohydrates and low molecular weight components that have many hydroxyl groups have

been studied, and their IRI activity was detected [7, 8]. It was found that IRI activity can correlate with the total number of hydroxyl groups in saccharide molecules [5]. Replacement of hydroxyl groups with hydrophobic alkyl radicals led to an increase in activity even if the total number of hydroxyl groups was reduced. This shows that although hydration is important, the presence of hydrophobic domains is also necessary to demonstrate IRI activity. It was reported [8] that PVA is a highly active IRI polymer, despite it has no obvious hydrophobic domains. However, according to its ability to increase the solubility of hydrophobic fluorescent dyes [3], PVA is capable to form hydrophobic domains in solutions.

The avoidance of crystal formation during the freezing and thawing courses is important for cell preservation during cryopreservation. It was found that during freezing, ice crystals can cause cell damage both mechanically and due to secondary physical and chemical phenomena caused by crystallization: increase in electrolyte concentration, cell dehydration, etc. [13]. PVA can exhibit IRI activity at the heating course, whereas other substances that are part of the cryoprotective medium may have not such properties [14].

The aim of this work is to study experimentally the IRI activity of PVA molecules of different length, and to find out its possible mechanism.

1. Materials and methods

We used polyvinyl alcohol with the molecular weight of 9 kDa (hereafter, PVA mol. wt. 9 kDa) (Aldrich, USA). PVA solutions were prepared on 0.1 M phosphate-buffered saline (pH 7.2) (hereafter, PBS) by weight method and expressed in wt%. Dissolution of PVA was carried out at 90 °C with constant stirring for 90 min. Solutions were examined the next day after their preparation. CCM for PVA was determined by stalagmometric method [15, 16].

Cryomicroscopic study of solutions was performed using an MBI-15 microscope, which was equipped with a cryo-adapter [17, 18]. This setup allows visual observation of the "water–ice–water" phase transition in the solution, the morphological state of the crystal structure in the process of cooling and heating. A drop of the studied PVA solution was placed on the slide glass of the cryo-adapter's working chamber. The sample was covered with a coverslip, which created a thin layer of the sample and eliminated the drying of the droplet. The samples were cooled at a rate of 1 °C/min to a temperature of –8 °C. The samples were heated at a rate of 1 °C/min. The temperature was controlled in the working chamber of the a cryo-adapter. Crystallization and melting kinetics of the samples were recorded using the Sigeta video camera in the frame-by-frame mode at a rate of 1 frame per 3 s. The temperature change in the working chamber of the cryostat corresponded to 0.05 °C for each snapshot.

To study the structural rearrangements of PVA molecules in solution, the 3-hydroxy-4'-(N,N-dimethylamino)flavone (DMAF) was used as a probe. This probe, as well as other flavonols, under illumination in solutions is capable to excited state intramolecular proton phototransfer (ESIPT) reaction, which results in two forms: normal (N^*) and tautomeric (T^*) [19–23], whose fluorescence band positions and intensities exhibits high sensitivity to the parameters of surrounding molecules. Thus, the degree of hydration of DMAF probe molecules in aqueous solution (i.e., the number of surrounding water molecules) can be characterized by the ratio of band intensities I_N^*/I_T^* [21, 24, 25]. DMAF was synthesized by the method described in [26].

Fluorescence spectra were recorded on a Cary Eclipse spectrofluorimeter (Varian, Australia) with a spectral slit width of 5 nm. The scanning step was 0.2 nm, and the accumulation time was 2 s. DMAF fluorescence was excited at wavelength of 405 nm in quartz cuvettes 1×1×3 cm at 22 ± 1 °C. Ethanol solution of DMAF was used in the experiments. The final concentration of the probe in the solutions was $1.7 \cdot 10^{-6}$ M. The positions of the maxima of the spectra were determined using the second derivatives.

Preferential conformations of PVA segments alone and their complexes with probes were modeled by optimizing the conformer energy using the MM+ molecular mechanics method [27].

2. Results and discussion

2.1. Study of crystallization of PVA solutions during freeze-thawing by cryomicroscopy

In the first stage, PBS was studied as a control solution (Table 1). When PBS was cooled in the temperature range (0 ... –1.45) °C the field of view remained stable, and when the temperature reached –1.50 °C the phase transition accompanied by the instantaneous formation of the crystal structure took place in the sample. In the temperature range (–1.50 ... –2.50) °C there was a gradual narrowing of the channels, in which the unfrozen fraction was localized, as a result of its partial freezing. Further cooling of the sample to –8 °C did not lead to changes in the morphology of the crystal structure that was formed. At the course of heating in the temperature range from –8.0 to –5.40 °C no significant changes in the crystal structure were observed. When the temperature reached –5.25 °C and up to –5.00 °C, the range of intense melting was observed. At –4.50 °C the crystal structure was completely melted and the field of view corresponded to the initial state (0 °C).

Table 1. Influence of PVA on the water–ice freezing temperature T_f and melting temperature T_m of the mixture in a PBS. ΔT_m is the melting interval width, determined visually by cryomicroscopy data

Solution	Freezing	Thawing	
	T_f, °C	T_m, °C	ΔT_m, °C
PBS	–1.5	–5.25	(–5.25 … –4.5)
+0.5% PVA	–1.35	–5.4	(–5.4 … –5.0)
+1% PVA	–1.4	–6.55	(–7.0 … –6.55)
+2% PVA	–1.85	–5.85	(–6.85 … –3.85)

When cooling the 0.5% PVA solution in the temperature range of (0... –1.30) °C, the field of view was unchanged. At –1.35 °C the phase transition was observed, which was accompanied by the formation of a crystal structure resembling a "dendritic" structure, which forms channels [Fig. 1(a)]. In the temperature range (–1.35 … –2.30) °C the gradual narrowing of the dendritic channels was observed due to freezing of the liquid phase and concentration of dissolved species in the channels. At the same time at –2.15 °C another, "needle-like" crystal structure appeared [Fig. 1(b)] on the background of the originally created crystal structure. During further cooling of the 0.5% PVA solution to –8.0 °C, the crystal structure retained its morphology, which indicates continuous freezing of the liquid fraction.

At the course of heating the 0.5% PVA solution in the temperature range from –8.0 to –6.0 °C, there was no significant change in the crystal structure. However, at the temperature of –6.0 °C dark inclusions appeared [Fig. 1(c)], which remained until the temperature of intensive melting (–5.40 °C). We assumed that the presence of these inclusions is due to the aggregation of PVA molecules, which were displaced out of the channels. Also in the area of intensive melting of ice crystals (–5.40 ... –5.0) °C an increase in the volume of the liquid fraction and the presence of "needle structures" were observed [Fig. 1(d)], which completely disappeared from the field of view when the temperature reached –4.95 °C. At the same temperature the ice melting process was completed and visually the state of

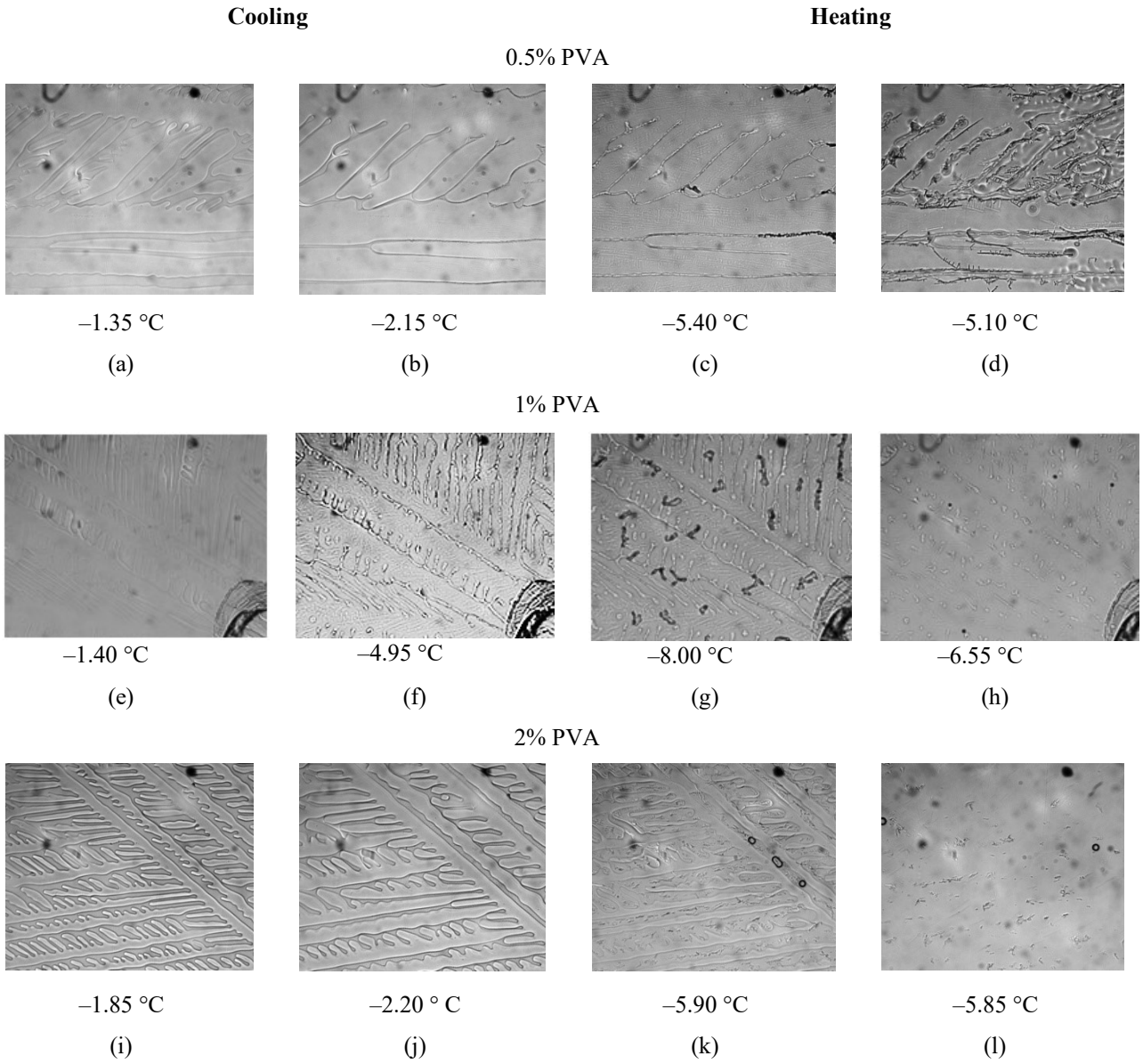

Fig. 1. Effect of cooling and heating on crystallization of PVA solutions (notes in the text).

the sample corresponded to the initial one (0 °C). The ice recrystallization process during the cooling and heating courses was not observed in the studied solution.

When 1% PVA solution was cooled, the field of view in the temperature range (0... –1.35) °C remained unchangeable, and when the temperature reached –1.40 °C, an immediate formation of the crystals separated by "dendritic" channels was observed [Fig. 1(d)]. In the temperature range (–1.40 ... –1.95) °C, freezing of the liquid fraction was observed, which was accompanied by narrowing of channels and an increase in the concentration of dissolved species in them. Decrease of the temperature to –4.90 °C did not lead to a significant change in the morphological structure of ice. At the temperature of –4.95 °C [Fig. 1(e)] appearance of dark inclusions was observed on the background of the originally formed crystal structure, their number insignificantly increased with further decrease in temperature. These inclusions had different shapes and sizes. Presumably, these inclusions represent PVA associates and other species displaced from the channels. Reducing the temperature to –8 °C did not affect the morphological structure of ice.

At the course of heating of 1% PVA solution at –8 °C no significant change of the crystal structure was observed, however the shape of the inclusions that appeared during cooling was changed [Fig. 1(f)]. In the temperature range (–7.0 ... –6.55) °C there was an intensive melting of the crystal structure and transformation of dark inclusions into round-shaped formations with dark edging. At –6.55 °C, the system was completely molten, but in the field of view we observed "needle-like" inclusions of an incomprehensible nature [Fig. 1(g)]. Possibly that these are salts of phosphate buffer, which precipitated out. When the temperature reached –6.45 °C, dark rounded inclusions disappeared from the field of view, but "needle-like" inclusions remained until the temperature of –4.5 °C. At this temperature the ice melting process was completed, but the visual state of the sample did not correspond to the initial one (0 °C). The ice recrystallization process during the cooling–warming courses was not observed in this solution.

When the 2% PVA solution was cooled, the field of view in the temperature range (0 ... –1.85) °C remained unchangeable. When the temperature reached –1.85 °C [Fig. 1(i)], the phase transition occurred with the formation of a crystals separated by wide "dendritic" channels. In the temperature range (–1.85 ... –2.50) °C there was freezing of liquid fraction, accompanied by narrowing of channels and increasing of concentration of dissolved species in them. At –2.20 °C [Fig. 1(j)] appearance of small particles in the grooves between the channels, which were more clearly visualized at lowering the temperature, was noted. They had a spherical shape but were of different sizes. Decrease in temperature to –8.0 °C did not lead to significant changes in the morphological structure of the ice.

At the course of thawing of the 2% PVA solution in the temperature range (–8 ... –6.85) °C no significant changes in the crystal structure were observed, however, the appearance of dark inclusions that changed their configuration with decreasing temperature was noted. When the temperature reached –5.90 °C [Fig. 1(k)] they had a spherical shape with a dark edging. At –5.85 °C [Fig. 1(l)] the melting of the crystal structure and the formation of "needle structures" were observed, which dissolved only when the temperature reached –3.85 °C. At this temperature, the visual state of the sample corresponded to the initial one (0 °C). The ice recrystallization process during the freeze-thawing courses in this solution was also not observed.

The studies showed that in all the studied PVA solutions with mol. wt. 9 kDa (0.5%, 1%, and 2%), no ice recrystallization was detected during the cooling-heating steps. Thus, the "dendritic" structure that emerged during the cooling of PVA solutions was more pronounced with increasing PVA concentration in the solution. However, it differed from the one emerged during cooling of PBS solutions, which resembled "straight" lines. It should be noted that in 0.5% PVA, a structure that resembled a "needle-like" structure was observed on the background of the originally formed structure. In the other studied solutions, this phenomenon was not observed. In 1% and 2% PVA solutions, the appearance of dark inclusions was observed both during the cooling and thawing courses, but when reaching a certain melting temperature, these inclusions completely disappeared.

Thus, the introduction of PVA changes the morphological structure of ice formed during cooling, shifts the freezing and melting temperatures, and suppresses recrystallization processes in the water–salt environment.

2.2. Study of the structural reorganization of PVA molecules in aqueous solutions

Figure 2(a) shows that in the PVA solution of mol. wt. 9 kDa, the DMAF probe has a two-band fluorescence spectrum. At PVA concentration of 0.1 and 0.2%, the maximum N^* band is at 515 nm, and the T^* band is observed as a shoulder. However, with increasing polymer concentration, it becomes separated band with a maximum at 565 nm. Both bands have close intensity similar to the form of DMAF spectra in solutions containing albumin or membrane structures [28].

As the PVA concentration in the aqueous solution increases [Fig. 2(a)], the intensity of the N^* and T^* bands of DMAF fluorescence increases non-monotonically. At the PVA concentrations of 0.1–1% there is a steep, above which, up to concentration of 5%, the fluorescence bands ratio insignificantly. The position of the N^* band maximum shifts to the short-wave direction, which corresponds to the increase in hydrophobicity of the probe microenvironment. Above the polymer concentration of 1%, the shift of the maximum has lesser amplitude.

Basing on the changes in the total fluorescence intensity and the position of the maximum N^* band, we assume that increasing the PVA concentration in the solution leads to

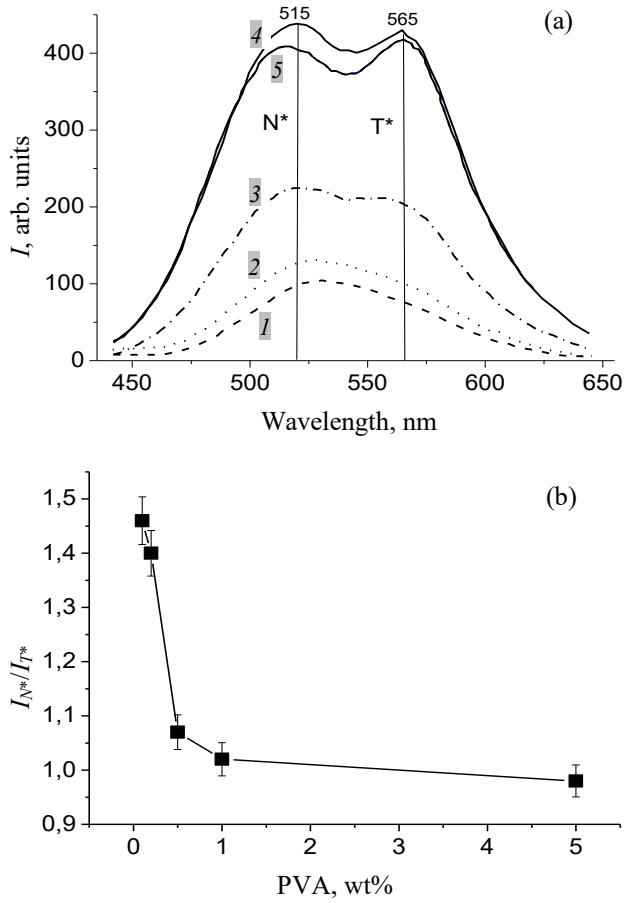

Fig. 2. (a) DMAF fluorescence spectra in PVA (9 kDa) solutions of various concentrations in PBS, pH 7.2: 0.1% (*1*), 0.2% (*2*), 0.5% (*3*), 1% (*4*), 5% (*5*); *I* is the fluorescence intensity, N^* is the normal form band, T^* is the tautomeric form band (λ_{ex} = 405 nm); (b) effect of PVA on the ratio of fluorescence peak intensities of the normal and tautomeric forms of DMAF.

the formation of a non-polar hydrophobic surrounding for DMAF molecules from which water molecules are gradually removed at the increase of PVA concentration.

The ratio of the fluorescence intensities of the I_N^*/I_T^* bands of DMAF [Fig. 2(b)] is an indicator of the number of water molecules in the probe microenvironment. In accordance with fluorescence intensity changes this parameter demonstrates steep decrease at 0.5–1% PVA concentrations and slow changes at higher values. This indicates a decrease in the total number of water molecules in the DMAF probe surroundings in the presence of PVA and confirms the conclusions drawn.

Summarizing the results, we can assume that in the concentration range of 0.5–1% PVA the composition of the DMAF probe solvation shell changes significantly, which reflects the changes of spatial structure of the polymer chain in solution with formation of cavities containing the probe. The rearrangement of the structure of the PVA solution in the above concentration range is also supported by a value of the CCM for this substance, which is equal to 0.24%.

Earlier published data also support our assumptions. The increase in the fluorescence intensity of the hydrophobic fluorescence probe diphenylhexatriene (DPH) was found [3] in the concentration range of PVA (9 kDa) above 0.5%. The authors explained this phenomenon by the formation of hydrophobic domains of polymer in the solution as a result of rearrangement of its spatial structure. Earlier it was noted that PVAs of high molecular weight are capable to form aggregates after freeze–thawing [29]. It was proposed that during the heating course the H-bonding system can be rearranged in aqueous PVA solutions [30], which affects the ability of the polymer OH groups to form intermolecular H bonds. The more conformationally mobile glycosurfactants, which have a low IRI, have similar hydrophobic domains, and their activity correlates with the chain number and hydration indexes. For example, octylglucoside, which has the large hydrophobic domain, was significantly less active than PVA. Above the CCM point (~25 μm [31]), octylglucoside has no hydrophobic domains because of the accumulation of homogeneous alkyl chains in the core. It is suggested [8], that this reveals PVA's unique ability to form hydrophobic domains either spontaneously or in response to the introduction of external molecules that disrupt its surface–active properties.

It has been experimentally shown [9], that such parameters as the molecular weight, the ratio of hydrophilic to hydrophobic sites, and the concentration in solution are important for the IRI activity of PVA. Thus, there is a certain critical chain length, exceeding which reduces the IRI activity of the polymer. It is assumed that a certain chain length is optimal for PVA interaction with the ice surface. Hydrophobic domains play an important role in the manifestation of IRI in proteins and their synthetic analogues, but in PVA molecules the orderly arrangement of hydrophobic groups leads to a decrease in IRI activity. That is, the molecular and spatial structure of ice recrystallization inhibitor in solution determine the efficiency of its adsorption on the ice particle surface, or the orientation of the polymer hydrophobic groups on this surface, which is important for the manifestation of IRI activity.

2.3. Molecular modeling of possible conformations of PVA chains

In order to study the possible spatial orientations of PVA chain segments in solution, in complex with DMAF probe and on ice nanoparticle surface, we performed molecular simulations of possible conformations of PVA chains alone as well as their DMAF probe aggregate. One of the obtained stable conformations [Fig. 3(a)] indicates the possibility of H-bonding of such segments of the PVA molecule to the ice particle surface directly or through the individual water molecules, which do not included to the ice surface. The high stability of such a complex is provided by the multiplicity of H bonds and their high stability. Its characteristic feature is the formation of hydrophobic covering on the

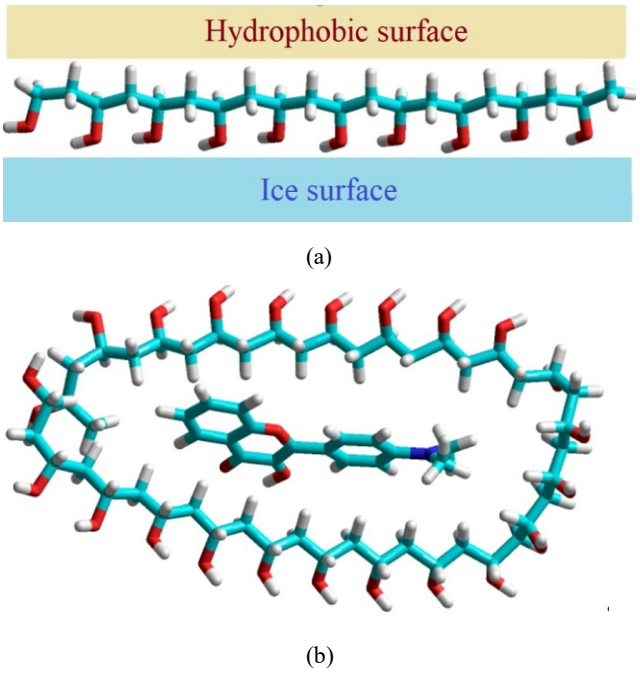

Fig. 3. Conformation of the PVA segment ($n = 10$), which demonstrates the possibility of binding to the ice crystal surface and formation of the hydrophobic surface on the outer side of the complex (a). Formation of a hydrophobic cavity by the PVA segment ($n = 21$) upon binding to the DMAF probe (b).

surface of ice particle. Such a non-polar hydrophobic covering by PVA molecules blocks the particle growth, since it screens the particle surface and reduces the degree of polarization of surrounding water molecules. By this it decreases an energy of their H bonds with the crystal particle surface. Hydrophobic proteins, the IRI activity of which was discussed above, can affect the ice surface in a similar way.

The formation of a hydrophobic surface by the segment of the PVA chain provides the formation of hydrophobic cavities in the surface of micelles, as it was predicted in [3]. Such cavities can be composed from the PVA segments depicted in Fig. 3(a), where one side is in H-bonding with water molecules, and the other one forms the walls of cavity. Since the PVA samples of higher molecular weight (15 and 31 kDa [8]) have lower ability for such conformational changes to provide the covering of ice particle surface, we assume that their lower IRI activity is the result of incomplete deployment and incomplete contact of long-chain polymers with ice surface.

Simulation of spatial structure of DMAF complex with the PVA chain segments showed that 16–20 monomeric chains from the two hundred available in PVA molecules (mol. wt. 9 kDa) are sufficient for complete shielding of the DMAF molecule from water. [Fig. 3(b)]. Such complex formation prevents the DMAF molecules from their aggregation in water. Based on the obtained results, we assume the possible mechanism for the IRI activity of PVA. The formation of PVA covering on ice crystal particles surface strongly changes the surface structure and properties. Thus, the PVA covering modifies the surface, depolarizing water molecules in close proximity, and inhibiting further growth of ice crystals.

Our results and assumptions are supported by the data obtained in other groups. It was shown by molecular dynamics simulations [32] that there is a spatial geometrical correspondence between the OH groups of PVA and ice molecules, and microscopic evidence of PVA adsorption on basal and prismatic faces of ice and incorporation of short-chain PVA into the ice lattice was provided. The authors suggested that the length of the PVA, i.e., the number of hydroxyl groups, seems to be the key factor determining the effectiveness of IRI, since the PVA molecule must be large enough to prevent neighboring warps from joining in the ice front.

As can be seen from our data, the process of crystal formation during freeze–thawing of a water-salt solution containing PVA (9 kDa), is quite complex and is not limited only to the interaction of polymer chains with ice. It is also capable to form local hydrophobic regions in aqueous solutions. Slow transition of conformations in the case of higher weight PVA samples affect its IRI activity, because they have lower ability for conformational changes. In the concentration range under study, the structure of water-polymer associates is rearranged, which can affect crystal formation and cell viability during the cooling and heating courses, when the concentration of dissolved components in the system changes as a result of incorporation to the ice particle of free and weakly bound water.

Conclusions

The processes of crystal formation and aggregation in aqueous solutions of PVA (mol. wt. 9 kDa). were studied using methods of cryomicroscopy, fluorescence probing, and molecular mechanics structure simulations. It was shown that in PVA solutions (0.5%, 1%, and 2%) the morphological structure of ice formed during cooling changes and ice recrystallization does not occur during the thawing course. It was assumed that in 0.1–5% aqueous solutions PVA undergoes rearrangements resulting in the formation of local hydrophobic areas and the formation of associates of the micellar type. At the PVA content > 3%, the size of PVA micelles increases due to their aggregation. The mechanism for the realization of the IRI activity of PVA polymers has been proposed, which consists in the formation of hydrogen-bonded complexes on the surface of ice nanocrystals. The formed complexes decrease the ability of further growth of ice crystals.

This work was supported by the National Academy of Sciences of Ukraine (research topic 2.2.6.102, supervised by Prof. A. M. Kompaniets).

Conflict of interest

The authors have disclosed no conflicts of interest.

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Механізм впливу полівінілового спирту (9 кДа) на утворення кристалів льоду у водних розчинах

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Запобігання кристалізації рідкої фази під час заморожування–відігріву клітин є однією з основних проблем, яку необхідно вирішити для успішного збереження біоматеріалу при низьких температурах. Одним із високоефективних інгібіторів рекристалізації є полівініловий спирт (ПВС). Однак механізми його кріопротекторної дії остаточно не з'ясовані. Зокрема, невідомо, які структурні особливості сприяють реалізації антирекристалізаційних властивостей ПВС у межах його кріозахисних концентрацій. Експериментально вивчено вплив ПВС на кристалізацію розчинника та структурні перебудови асоціатів молекул ПВС у натрій-фосфатному буфері (НФБ). Досліджено розчини ПВС (молекулярна вага 9 кДа) у НФБ за допомогою методів кріомікроскопії та флуоресцентної спектроскопії. Показано, що молекулярні асоціати ПВС у НФБ зазнають перебудови близько 0,5–1 ваг.%, яка супроводжується зміною їх розміру та гідрофільно-гідрофобних властивостей. ПВС також змінює морфологічну структуру льоду при охолодженні та перешкоджає кристалізації при нагріванні. Припускається, що механізм антирекристалізаційної активності ПВС може обумовлюватися утворенням його комплексів з поверхнею кристалів льоду.

Ключові слова: полівініловий спирт, кристалізація, структурні перебудови, флуоресценція.