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FOCUS REVIEW

Bridging polymer chemistry and cryobiology

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Abstract

Polymers, especially charged polymers, are the key to a sustainable future, as they have the capability to act as alternatives to plastics, reduce the impact of global warming, and offer solutions to global environmental pollution problems. Biomaterial polymers have proven to be incredibly effective in a multitude of applications, including clinical applications. In the fields of cryobiology and cryopreservation, polymers have emerged as credible alternatives to small molecules and other compounds, yielding excellent results. This review outlines the results of research in the areas of polymer chemistry and cryobiology, which have not been discussed together previously. Herein, we explain how recent polymer research has enabled the development of polymeric cryoprotectants with novel mechanisms and the development of novel methods for the intracellular delivery of substances, such as drugs, using a cryobiological technique called the freeze-concentration effect. Our findings indicate that interdisciplinary collaboration between cryobiologists and polymer chemists has led to exciting developments that will further cell biology and medical research.

Introduction

Polymeric materials are used in various fields owing to the ease of adding functionality through molecular design. For example, in the biomedical field, polymers are used as surface coatings for implantable materials [1, 2], socket materials for artificial joints [3], substrates for drug delivery systems [4, 5], scaffolds for tissue engineering or transplantation [6], and optical materials such as contact lenses and intraocular lenses [7]. In the field of regenerative medicine, many studies are being conducted on polymers and polymeric hydrogel materials that can be used as scaffolds for cell cultures [8]. Polymer chemistry involves fundamental and applied knowledge that can be used not only in the field of biotechnology but also in materials-based fields, such as the energy and electronics fields.

For example, in the field of cell biology, which is essential for medical biology research, it was reported that poly(vinyl alcohol), one of the simplest polymers, has shown potential for the control of cell differentiation [9]. It is very exciting to see new reports of simple polymers manifesting unexpected biological activities. Polymers have also found a niche in the field of cryobiology, which was previously dominated by small molecules. Among the cryoprotective polymers discussed in this review are some with surprising properties.

In the field of cell research, techniques have already been established for the freezing and preservation of cells for delivery and storage. The technology is based on the protective effect of adding a cryoprotectant (CPA) to the cells. By adding a small-molecule CPA, such as dimethyl sulfoxide (DMSO), and keeping the cells at a low temperature, the cells can be protected from damage during freezing [10]. Although polymers have not often been applied for such purposes, increasing pressure to avoid the use of DMSO, which is cytotoxic and affects cell differentiation [11], has prompted research into polymeric CPAs that do not penetrate cell membranes. DMSO remains the preferred CPA in many biological applications because there are no effective substitutes. We were the first to report that polyampholytes (polymers containing positive and negative charges) have cryoprotective properties for cells [12]. Moreover, we studied polyampholytes to determine the relationship between their molecular structure and their function, elucidated their mechanisms, and investigated their applications [13–16].

Cryobiology involves the study of the behavior of biological systems at low temperatures, including the dynamics of biomolecules, cells, and organisms during freezing [17]. When a cell suspension is frozen, the extracellular fluid

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freezes first, and then the solutes excluded from the ice crystals are concentrated in a process called freeze concentration [18]. This dehydrates the cells and prevents the lethal formation of intracellular ice, which is one mechanism for avoiding freezing damage. In this case, the addition of materials such as micelles, nanogels, nanoparticle-carrying proteins, and other substances to cells prior to freezing increases their concentration in the solution around the cells, making it possible to concentrate the materials around the cells simply by freezing and thawing. Moreover, by using polymeric carriers with a high affinity for cell membranes, it is possible to increase the efficiency of material transfer into cells by adsorption to the membrane and automatic uptake by the cells [19, 20].

The content of this review bridges the fields of polymer chemistry and cryobiology and includes the development of cryoprotective materials using polymer chemistry technology and intracellular drug delivery systems constructed from polymeric materials based on the phenomenon of freeze concentration, specifically those using polyampholytes as polymeric CPAs. Additionally, the mechanism of cryoprotection is discussed in detail. Polymeric compounds are more adaptable and versatile than their small-molecule counterparts and have the potential to revolutionize the development of newer and more efficient CPAs.

Polymeric cryoprotectants—polymer chemistry and cryobiology

Cells required for medical biological research are often stored frozen for long periods and thawed for use as needed. In such cases, the addition of a CPA is generally required to prevent cell death during freezing. Glycerol was first reported as a protective agent by Polge et al. in 1949 [21], and 10 years later, DMSO was reported to be effective for the cryopreservation of red blood cells [10]. DMSO is used as a CPA in a wide range of cell cryopreservation applications, such as cell banking and cell cryopreservation in laboratories, because of its low cost and high cryoprotective activity. However, alternative CPAs are sometimes required to replace DMSO, such as in cases in which its toxicity to cells or organisms cannot be ignored or cases involving cells whose differentiation is affected by DMSO [11]. In recent years, various compounds ranging from small molecules to polymers have been reported as new CPAs to meet such needs [22].

Small-molecule CPAs can be divided into membrane-permeable and nonpermeable CPAs. Glycerol and DMSO are representative membrane-permeable CPAs. Glycerol is currently used as a CPA for red blood cells, fertilized eggs, and sperm from livestock. Glycerol is a polyol, a compound with multiple hydroxyl groups. DMSO is a CPA used in a wide range of fields, including the cryopreservation of various types of cells in cell banks and cryopreservation of cells in laboratories. These CPAs are reported to suppress the formation of intracellular ice crystals by penetrating cells and dehydrating and displacing cells [23]. In contrast, sugars, including disaccharides such as trehalose and sucrose, and oligosaccharides, such as raffinose, are typical examples of membrane-impermeable CPAs. These sugars displace water in the vicinity of the cell membrane and protect the membrane [24]. The polysaccharide hydroxyethyl starch (HES) has also been used as a CPA [25]. It is predominantly used as an adjunct to membrane-penetrating CPAs such as DMSO. Recently, ionic liquids [26] and glycolipids [27] have been reported to have cryoprotective properties, and the search for CPAs from the viewpoint of molecular chemistry using computer simulation has attracted significant interest [28].

A wide range of substances are being investigated as macromolecular CPAs, including naturally occurring substances such as proteins and polysaccharides, as well as synthetic macromolecules. For example, cell cryoprotective effects have been reported for proteins extracted from wheat [29]. Moreover, several polymer-based CPAs (including polyampholytes) that demonstrate remarkable properties have been developed, and the structures of several synthetic polymer-based CPAs are summarized in Fig. 1, with further details provided in Table 1. Various synthetic polymers have been reported to exhibit cryoprotective effects, and interesting studies have been conducted on the correlation between structure and function. Notably, polymeric CPAs are hydrophilic and often contain a number of charges or introduced sugar moieties. Herein, we have classified these polymeric CPAs in terms of their mechanisms.

Proposed mechanism of ice crystal formation inhibition effect

Polyvinylpyrrolidone, polyvinyl alcohol (PVA), and some proteins have been reported to act as cryoprotective polymers, and they are often used in combination with small-molecule protectants. Antifreeze proteins (AFPs) are a class of proteins obtained from many plants and animals, including fish, insects, and fungi, that can survive under freezing conditions. They have been reported to inhibit ice crystal formation by binding to ice nuclei [30, 31]. Research is underway to realize cryopreservation using AFPs or to mimic them via polymeric compounds. Gibson et al. developed CPAs based on synthetic polymers by utilizing the inhibitory effect on ice crystal formation [32, 33]. For example, by changing the degree of saponification and degree of polymerization of PVA, which has many

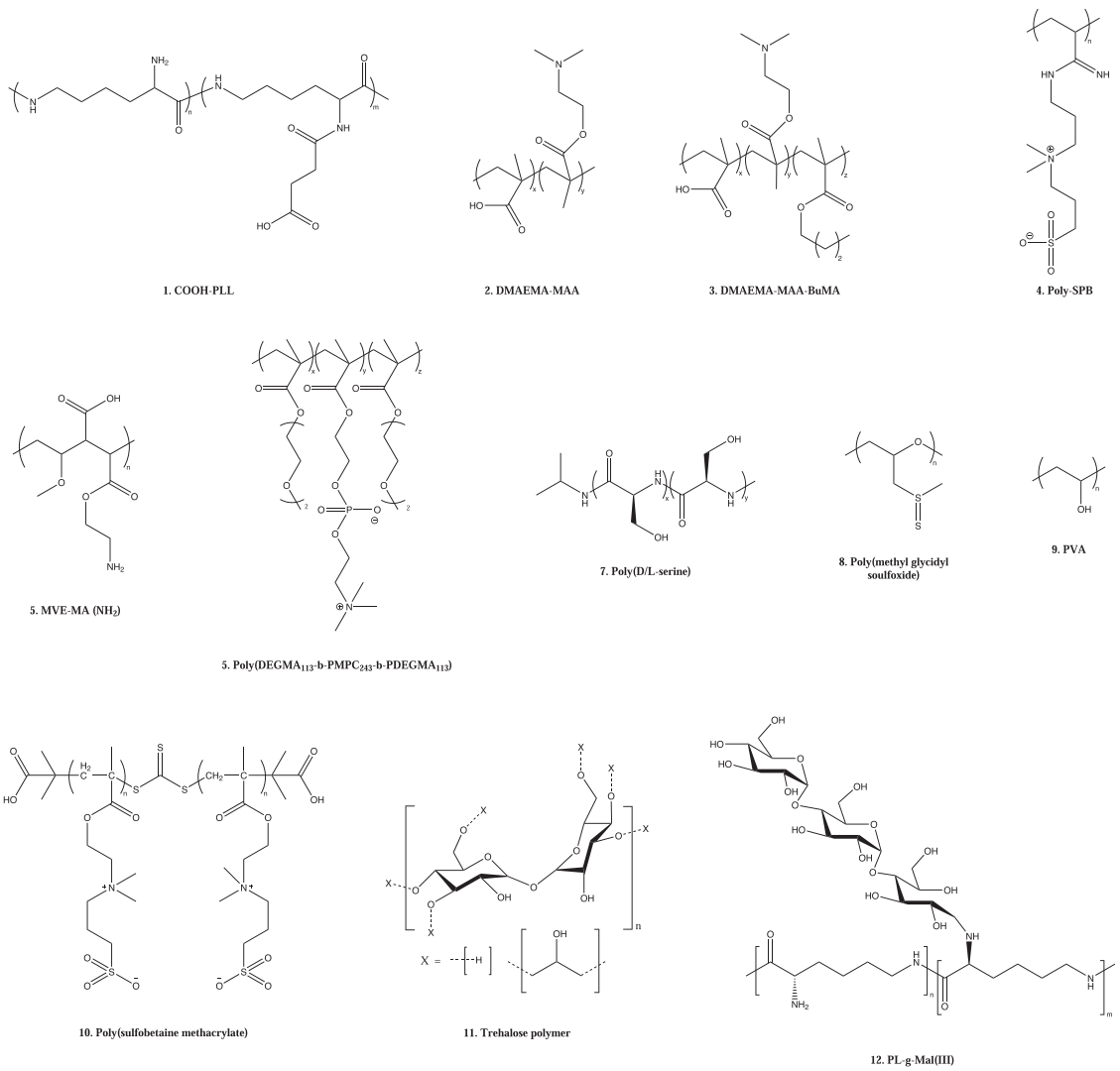

Fig. 1 The structures of various polymeric cryoprotectants are listed in Table 1

hydroxyl groups with high binding affinities for ice crystal surfaces, they developed a polymer with a high inhibitory effect on ice recrystallization and used it for the cryoprotection of red blood cells [32]. The strong IRI activity is reported to be caused by hydrogen bonds between PVA and ice, which increases the enthalpic gains and raises the entropic factor as a result of the desolvation of the methylene groups of PVA [34].

Ben et al. chemically modified an AFP and succeeded in enhancing the cryoprotection of cells [35]. As the formation of ice crystals causes physical damage to cells, it is clear that the inhibition of ice crystal formation plays an important role in cell viability during freezing. The inhibition of recrystallization during thawing is also necessary, and there are reports that high survival rates have been obtained even under slow thawing conditions with the addition of PVA [33]. The addition of PVA to erythrocyte suspensions suppressed ice crystal growth during freezing and thawing, and hemolysis was suppressed relative to that in the system with no PVA added (buffer alone; Fig. 2). However, the authors of some studies have reported no cryoprotective effect of AFP, and the cryoprotective effect cannot be explained by the inhibition of ice crystal formation and growth alone [36].

The cell membrane protection effect is important for preventing damage to the membrane caused by ice crystals when the cells are frozen. For example, trehalose polymers have been synthesized to enhance the water-substitution effect of trehalose [37, 38]. In addition to the inhibition of ice crystal formation, cell membranes are reported to contribute significantly to cell cryopreservation.

Rajan et al. used electron spin resonance to confirm that the introduction of alkyl chains to polyampholytes (Fig. 1, structures 2 and 3) enhances their interaction with cell

Table 1 Cryoprotective outcomes using various polymer cryoprotectants

Structure	Name	Cell type	Cell viability/%	Polymer concentration/%	Other CPAs[a]	Ref
1	COOH-PLL	L292, MSCs	~95	7.5	None	[12]
2	DMAEMA-MAA	L929	~90	15	None	[15]
3	DMAEMA-MAA-BuMA	L929	~95	10	None	[15, 39]
4	Poly-SPB	L929	~70	15	None	[39]
5	MVE-MA(NH_2)	A549	50	2	5% DMSO[b]	[58]
6	Poly($DEGMA_{113}$-b-$PMPC_{243}$-b-$PDEGMA_{113}$)	CHO, PC3, HeLa, FaDu, Fibroblasts, K562, W138	~95	3–15	None	[59]
7	Poly(D/L-serine)	Red blood cells	50	6	None	[60]
8	Poly(methyl glycidyl sulfoxide)	3T3, Fibroblasts	60–80	10	None	[42]
9	PVA[c]	Red blood cells	60	0.1	21.5% HES[d]	[32]
10	poly(sulfobetaine methacrylate)	Chondrocytes	80	1	4% betaine	[61]
11	Trehalose polymer	HeLa. Fibroblasts	90	5–10	10% DMSO + 0.5 M trehalose	[37]
12	PL-g-Mal(III) glycopeptide	Erythrocytes	74	0.4	0.36 M trehalose	[62]

[a]Cryoprotectant.

[b]Dimethylsulfoxide.

[c]Polyvinyl alcohol.

[d]Hydroxyethyl starc.

membranes and reported a relationship between hydrophobicity and protective effects (Fig. 3) [39]. Stöver et al. reported an improvement in the protective activity of polyampholytes in which a hydrophobic *t*-butanol group was introduced [40]. Moreover, the introduction of hydrophobicity was found to enhance the recrystallization inhibitory effect, which may have a synergistic effect of preventing ice crystal damage in the vicinity of the cell membrane.

Proposed mechanism—intracellular ice crystal inhibition effect

Intracellular ice crystal formation is a problem that should be considered when freezing cells. Intracellular ice crystals are reported to cause irreversible damage to intracellular organelles, resulting in cell death. Cell membrane-permeable CPAs such as DMSO are thought to inhibit ice crystal formation and reduce ice crystal size by penetrating cells and replacing water. Moreover, most polymeric CPAs do not penetrate cell membranes [12]. Our group showed that carboxylated polylysine (carboxylated poly-L-lysine, COOH-PLL) has a cell cryoprotective effect. However, the mechanism of this effect has not been clarified. In 2021, we reported that the regulation of cell dehydration is an important mechanism for the inhibition of intracellular ice crystal formation [41].

The mobility of water molecules and salt ions in aqueous solutions of COOH-PLL between 0 and –41 °C was evaluated using solid-state NMR measurements. The results showed that the mobility of water at low temperatures was significantly suppressed and the viscosity increased in the COOH-PLL solution compared to other polymers or DMSO solutions. Under freezing conditions, the polymer solution surrounding the cell has a high viscosity, suggesting that it inhibits the formation of intracellular ice crystals through the penetration of ice crystals into the cell. The polymer chains trap Na ions and reduce their mobility at low temperatures (Fig. 4a). This reduces the concentration of Na ions that contribute to osmotic pressure, suppressing rapid dehydration and achieving optimal conditions for sufficient intracellular dehydration under mild conditions, suggesting that the formation of intracellular ice crystals is suppressed (Fig. 4b). Lynd et al. reported a polymeric protectant that inhibits intracellular ice crystal formation by successfully controlling cell dehydration [42], an interesting finding that may guide the molecular design of future CPAs.

This effect can be described as the inhibition of crystallization because the increase in viscosity due to the enhanced interaction between the polymer and water at low temperatures inhibits the molecular motion of water. Therefore, designing the structure of the side chains and main chains of polymers may contribute to greater ice crystallization inhibition and enhanced dehydration. For example, we have reported that the addition of similar

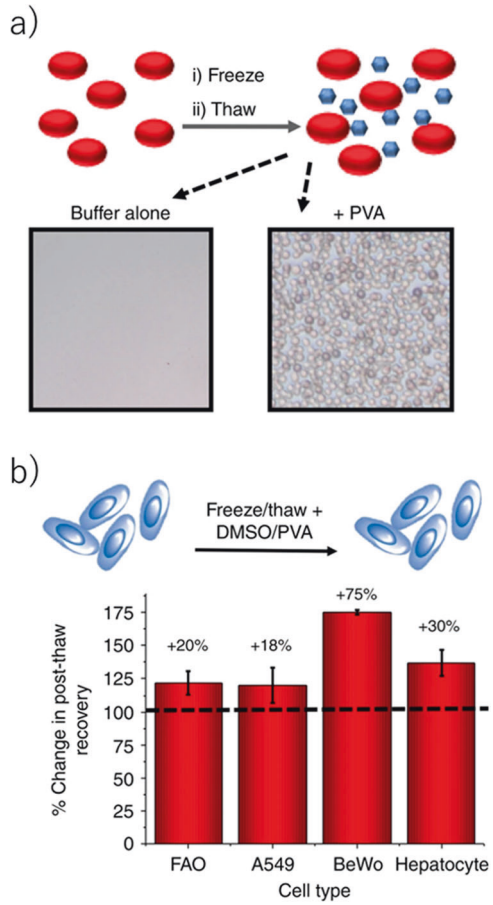

Fig. 2 Cryopreservation of cells using poly(vinyl alcohol) (PVA). **a** Solvent-free red blood cell cryopreservation upon addition of 1 mg mL^{-1} PVA. Micrographs show recovered intact red blood cells; (**b**) somatic cell recovery postthaw in PVA/dimethyl sulfoxide (DMSO) mixtures. Reproduced with permission from ref. [33], under Creative Commons CC-BY license

zwitterionic polymers suppresses the thermal aggregation of proteins [43, 44], and we believe that the protective effect of dehydration inhibition is also involved. In conclusion, we have outlined the role of structurally engineered polymer CPAs in the new field of cryobiology.

Cryobiology and polymer materials science of intracellular substance transport by freeze concentration

It was long believed that the application of cryopreservation was limited to preserving biological specimens only. However, recent reports reveal that this technology can be useful in a multitude of fields, ranging from cryobiology to polymer chemistry and materials science (biomaterials) research.

The intracellular transport of substances has attracted much attention as a drug delivery technique [45]. The cell membrane acts as a barrier between the inside and outside of the cell and prevents the permeation of various substances. For example, to introduce nucleic acids into cells for gene therapy, a complex must be formed with cationic phospholipids or polymers that interact with the negative charge of the nucleic acids [46]. In addition, cationic vehicles are generally highly cytotoxic, so molecular design is important. The cellular delivery of proteins is also useful for the intracellular expression of protein drug effects and immunotherapy by antigen delivery. For such intracellular transfection, it is necessary to devise a way for the cargo to escape from endosomes to prevent degradation by endosomes and lysosomes after uptake by cells through endocytosis [47]. However, methods of cytoplasmic translocation that do not involve endosomes, such as membrane-permeable peptides, have also been developed [48]. In addition, the introduction of medium-sized molecular drugs such as oligopeptides into cells for the control of protein–protein interactions has recently attracted considerable interest [49].

Freeze concentration

In the food industry, concentration and reduction are commonly used to prepare juices and other products. However, the aromatic components evaporate during the drying process. This has led to the development of the freeze-concentration method [50, 51]. This method increases the concentration of juice through the exclusion of solute from the ice crystals that are formed when an aqueous solution is slowly frozen, thereby concentrating the remaining water. After freezing, the concentrated liquid can be extracted by slow thawing so that the concentrated portion dissolves first. For example, when a pH 5 solution of bromophenol blue solution, a pH indicator, is frozen, the blue color indicating pH 5 shifts toward of the color indicating pH 3, as shown in Fig. 5. This indicates that the protons have been concentrated 100-fold. This technique has also been used to promote chemical reactions that are concentration dependent. For example, click chemistry and the improvement of the reactivity between polymers and small nucleic acids by freezing have been reported [52]. Miyawaki et al. reported that this freeze-concentration effect can be easily obtained from the melting point [53].

Theoretically, the freeze-concentration factor, α, which represents the degree of concentration by freezing, is related to the freezing-point depression and was estimated from the analysis of the fraction of frozen water.

Specifically, the freeze-concentration factor (α) is described as

$$\alpha = \frac{freezable\ water}{unfrozen\ water} = \frac{F_{fw}}{F_{fw} - F_f} \quad (1)$$

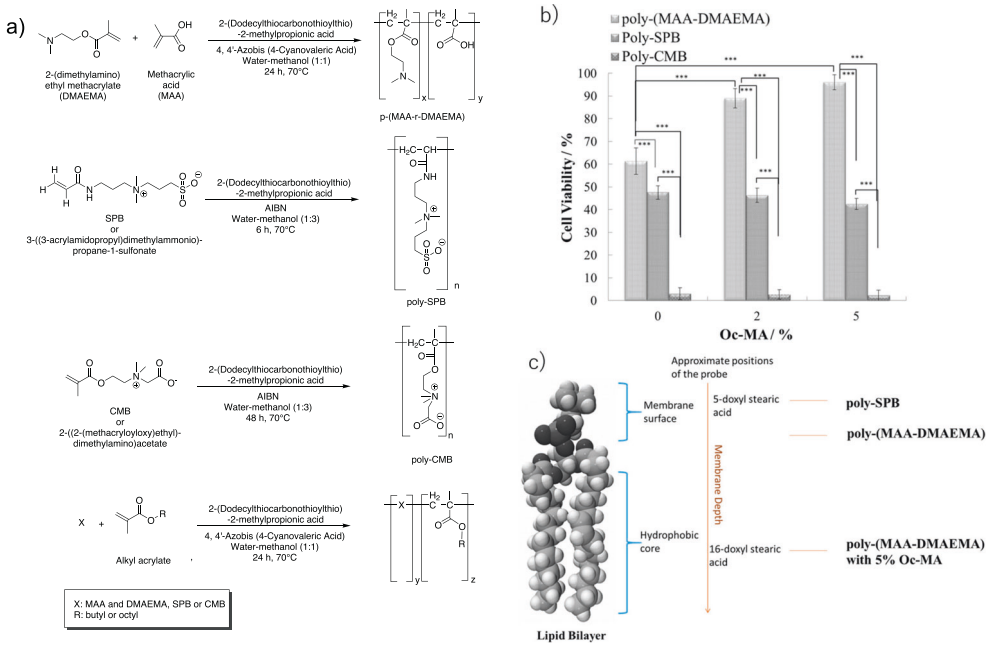

Fig. 3 **a** Synthesis of poly-(MAA and DMAEMA), poly-SPB, poly-CMB, and hydrophobic derivatives of polyampholytes by RAFT polymerization. **b** Cryoprotective properties of poly(MAA-DMAEMA), poly-SPB, and poly-CMB with different OcMA concentrations at a constant polymer concentration of 10%. L929 cells were cryopreserved with different polyampholytes at various concentrations. Data are expressed as the mean ± SD for three independent experiments (five samples each). ***$P < 0.001$. **c** Schematic representation of membrane–polyampholyte interaction/localization. Reproduced with permission from ref. [39]

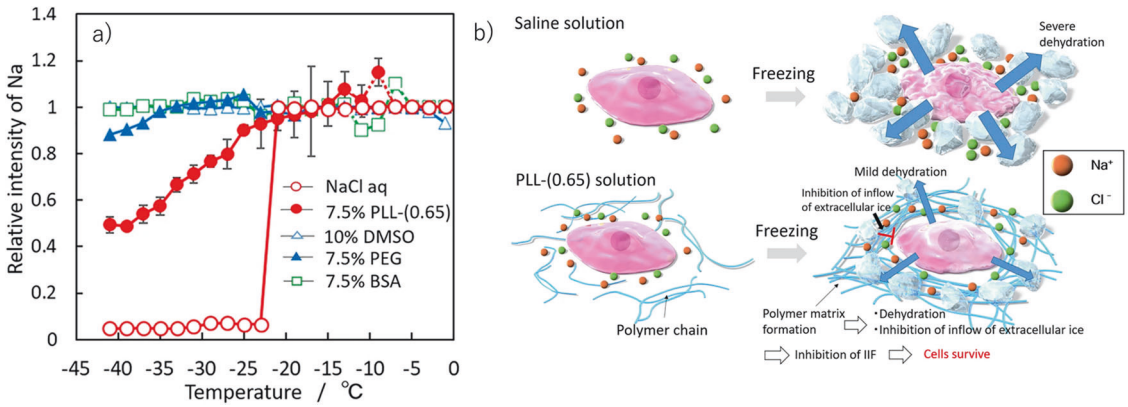

Fig. 4 **a** Temperature dependence of Na-ion signal intensities. The relative intensity values of the polymer solution in (**a**) above −15 C are connected by dotted lines because the two components are not easily separated and there is a large degree of variation. Error bars represent the standard deviation. **b** Schematic illustration of the mechanism of cryoprotection by COOH-PLL (PLL-(0.65)). Reproduced with permission from ref. [41], under Creative Commons CC-BY license

where *Ff* is the fraction of frozen water in the total water (g of frozen water/g of total water), and *Ffw* is the fraction of freezable water determined by the equation

$$F_{fw} = \frac{X_w - X_{uf}}{X_w} \tag{2}$$

where *Xw* is the water content (g of water/g of total mass) and *Xuf* is the unfreezable water content (g of water/g of total mass).

The *Xuf* was measured using differential scanning calorimetry, and the fraction of frozen water in the freezable water (g of frozen water/g of freezable water) at a particular temperature *T* (°C) is described by the following equation.

$$\frac{F_f}{F_{fw}} = \left(1 - \frac{T_f}{T}\right) \tag{3}$$

where *Tf* is the freezing point of the system (°C).

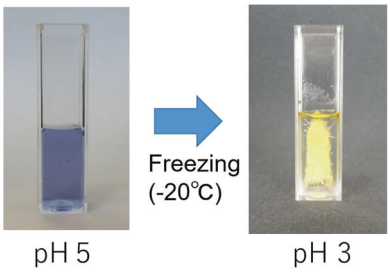

Fig. 5 Freeze concentration of bromophenol blue solution. The color change indicated that protons were concentrated 100 times by simple freezing

The unfrozen water fraction in the freezable water can be obtained using

$$\frac{F_{fw} - F_f}{F_{fw}} = \frac{T_f}{T}. \quad (4)$$

After combining Eqs. (1) and (4), a simple equation for determining the temperature-dependent freeze-concentration factor with only one parameter, *Tf*, can be derived as follows:

$$\alpha = \frac{T}{T_f}. \quad (5)$$

From these equations, we determined the freeze-concentration factor of a cell suspension with 10% DMSO as a CPA and 10% COOH-PLL, as described above (Fig. 6) [54]. Notably, the freeze-concentration factor was clearly higher for the polymeric system than for the DMSO system. This is because the molar concentration of the polymeric system is lower, and therefore, freezing-point depression is less likely to occur and the degree of concentration is higher, as indicated by the calculation results. Therefore, our polymeric CPA can dramatically increase the concentration of solute in the remaining solution around the cell.

For the intracellular introduction of proteins, we loaded the proteins onto polymeric nanoparticles and liposomes with high affinities for cell membranes and allowed them to adsorb onto the cell membranes via freeze concentration [19, 20]. The liposomes were coated with polyampholytes to allow their escape from the endosomes after cellular uptake. For example, liposomes encapsulated with fluorescent-labeled lysozymes were added to a cell suspension, frozen as a CPA, and then thawed. Lysozyme was adsorbed onto a large area of the cell surface simply by freezing. After 24 h, the migration of proteins into the cells was confirmed (Fig. 7a). Escape most likely occurred because liposomes coated with polyampholytes aggregated in the favorable pH environment in the endosomes, destabilizing the membrane (Fig. 7b). Thus, we have reported a simple technique for freeze-thawing that allows macrophages to efficiently ingest antigens [55], introduce genes [56], and perform cell imaging by introducing quantum dots

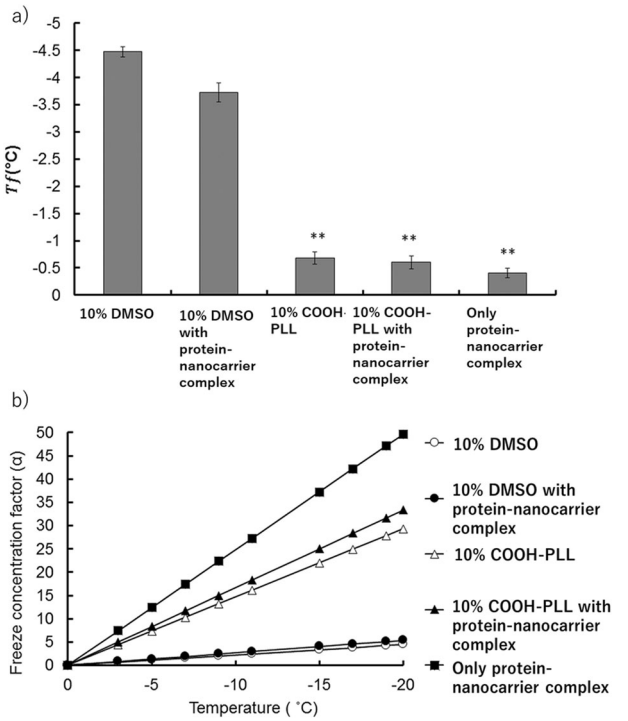

Fig. 6 Estimation of the freezing point (*Tf*) and freeze concentration factor (α) during freezing. **a** Determination of the freezing point of 10% DMSO with or without the protein–nanocarrier complex, 10% COOH-PLL with or without the protein–nanocarrier complex, and the protein–nanocarrier complex without cryoprotectant. **b** On the basis of the freezing point of the respective samples, the freeze concentration factor (α) was calculated at the following temperatures: 0, −3, −5, −7, −9, −11, −15, −17, −19, and −20 °C. The graph shows the freeze concentration factor plotted against temperature. Data are expressed as the mean ± SD, $^{**}p < 0.01$. Reproduced with permission from ref. [54]

[57]. This method is inexpensive, novel, and does not require special equipment.

Conclusions and future perspectives

In summary, we have reviewed research across two fields and discussed the solutions to problems in cryobiology from the viewpoint of polymeric materials science and the applications of polymer-based cryobiology for biomedical applications.

The advantage of polymer-based CPAs is that the molecular weight, steric structure, and side-chain structure can be controlled, making it possible to optimize the functions associated with structural changes. For example, it is easy to form a hydrogel by cross-linking, and the use of hydrogels as scaffolds for cell cultures after thawing has been reported. Although polymers (especially polyampholytes) have been used to advance technology over the past few decades, the field of polymer-based CPAs is still in its infancy, and more attention and focused research are

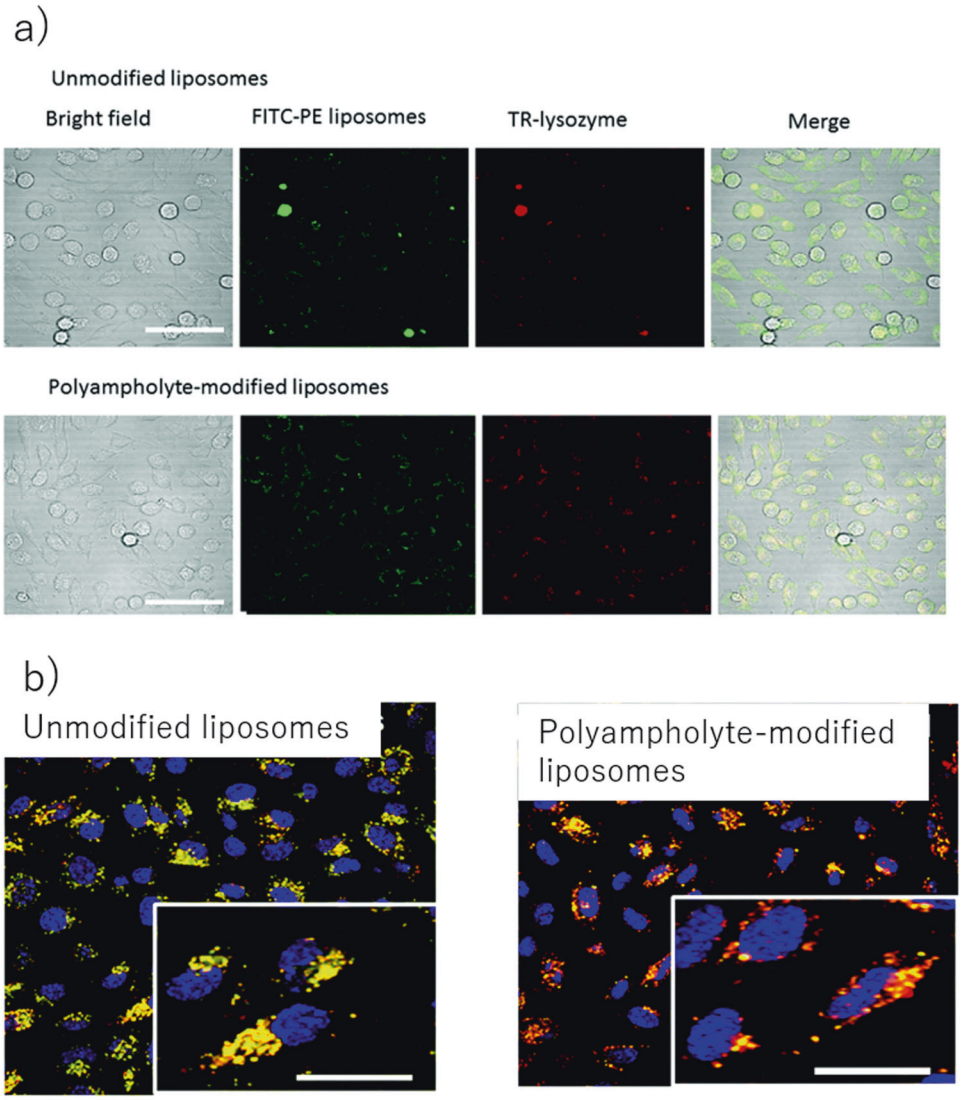

Fig. 7 Confocal microphotographs of L929 cells. **a** The images show that lysozyme protein internalization occurs via endocytosis during culture after being frozen with lysozyme-loaded modified liposomes using 10% COOH-PLL as a cryoprotectant. (upper) Unmodified liposomes; (lower) polyampholyte-modified liposomes. Scale bars: 50 µm. **b** Intracellular delivery of TR-labeled lysozymes in L929 cells. We cryopreserved 1×10^6 cells with the polymeric cryoprotectant COOH-PLL and protein-containing liposomes. The cells were thawed and seeded for 12 h at 37 °C. After incubation, the endosomes/lysosomes and nuclei were stained with LysoTracker Green and Hoechst blue 33258, respectively. (left) Unmodified liposomes. (right) Polyampholyte-modified liposomes. Scale bar: 10 µm. Reproduced with permission from ref. [20]

required to address the current drawbacks and hasten the widespread application of polymer-based CPAs. The optimization of molecules by molecular dynamics simulation has been studied, and it is expected that new research fields, such as the creation of new data-driven CPAs through machine learning, will evolve in the future.

Currently, the boundaries between research fields are less clear, and exchanges between researchers from different fields have opened up new pathways for research that would have been unthinkable without cross-disciplinary collaboration. It is exciting to see new academic fields evolve as a result of bringing together knowledge from different fields.

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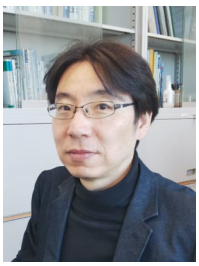

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