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The basic principles of cryopreservation and the importance of polyampholytes as a cryoprotectant

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ABSTRACT

Cryopreservation is an important process used to store the materials such as biological samples and food in liquid nitrogen (-196 °C) or in ultralow temperature freezer (-86 °C) for various biomedical, clinical and food-related applications. The main goal in the cryopreservation is to protect the materials against to damages during the freeze-thaw steps. Polyampholytes, which are the polymers containing both the cationic and anionic groups, have emerged as promising cryoprotective agents due to their unique properties. This review comprehensively discusses the history of cryopreservation, its fundamental principles, and the synthesis and cryoprotective properties of polyampholytes, with a focus on their mechanisms and applications.

Keywords: polyampholytes, cell cryopreservation, cryoprotectant, non-permeable cryoprotectant.

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Introduction

Cryopreservation is a crucial technique in regenerative medicine, tissue engineering, and biobanking (Pegg and Karow, 1987). The ability to store biological materials for extended periods while preserving their viability, properties and functions is essential for various applications, including transplantation, drug discovery, and basic research. However, the formation of ice crystals during freeze-thaw procedures can cause severe cellular damage, limit the success of cryopreservation (Mazur, 1970).

To minimize ice crystals formation, the cryoprotective additives (CPAs) called as cryoprotectant such as glycerol and dimethyl sulfoxide (DMSO) are very important agents in the traditional cryopreservation methods (Fahy, 2014). These CPAs penetrate cell membranes, reduce intracellular ice formation and osmotic stress (Hubel, 2009). However, they can be toxic at high concentrations and may not be suitable for all cell types. Therefore, there is growing interest in developing novel CPAs with enhanced cryoprotective efficacy and reduced toxicity (Yuan et al., 2024). Polyampholytes, which contain both cationic and anionic groups, have emerged as promising CPAs due to their unique properties. These polymers can interact with cell membranes, prevent ice crystals formation, and reduce osmotic stress (Stubbs et al., 2020b). This review provides a comprehensively overview of the history of cryopreservation, fundamental concepts, and the synthesis and cryoprotective properties of polyampholytes, with a particular focus on their mechanisms of action and potential applications.

History of cryopreservation

The initial observation of spermatozoa maintaining motility at low temperature conditions was documented by Lazzaro Spallanzani in 1776, following

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the invention of the compound microscope (Walters et al., 2009). The origins of research into the effects of cryopreservation on living tissues can be traced backed to the late nineteenth century, when scientists began using this technology to preserve spermatozoa, somatic cells and red blood cells. During this period, studies revealed inherent limitations in the cryopreservation process, resulting in inconsistent outcomes and frequent instances of infertility attributable to early embryonic mortality.

In 1953, James Lovelock demonstrated that the rapid freezing of liquid components during cryopreservation leads to ice crystal formation, which in turn causes osmotic stress within cells. Mazur (1963) successfully characterized this mechanism by demonstrating that the rate of temperature change in the medium containing cells governs the movement of water across cellular membranes, thereby determining the extent of intracellular freezing. These findings collectively enhanced the understanding of the mechanisms associated with the cryoprotective process.

Research conducted in the 1980s noted that the cooling and warming speed of both freezing and thawing were a crucial determinant of cellular viability (Al-Hasani et al., 1987; Chesné and Guillouzo, 1988) It was demonstrated that gradual, incremental changes during these processes prevented the rapid formation of ice crystals, which would otherwise increase membrane-bound solute concentrations and lead to premature cellular death (Mazur et al., 1972). A significant advancement in cryopreservation presented in the late of the 1940s was that when the cryopreservation of spermatozoa was realized in medium containing glycerol, which is a polyol and membrane-permeable cryoprotectant, at -70 oC, the viability rate of spermatozoa improved (Polge et al., 1949). During the cryopreservation process, adding the glycerol to the medium effectively protected the cells against to the rapid formation of ice crystals. It was reported that DMSO as a membrane-permeable cryoprotectant such as glycerol was also commonly used in the freezing process (Friedler et al., 1988; Siebzehnruebl et al., 1989). When dimethyl sulfoxide (DMSO) is introduced into the cell culture medium at a concentration of 10% (typically corresponding to approximately two molar), it enhances cell membrane permeability, allowing water to move more freely across the membrane.(Boquet et all., 1995; Ock and Rho, 2011). Besides, it was also reported that like glycerol, DMSO inhibited the formation of ice crystals by increasing intracellular solute concentration, thereby promoting water vitrification at low temperatures (Mandumpal et al., 2010).

Fundamental concepts of cryopreservation

Cryopreservation is a process in which cells, tissues, organs or other biological structures are cooled to very low temperatures such as -196 °C, thus stopping all biological activity and preserving them for future use with minimal damage and loss of their functions (Özkavukçu and Erdemli, 2002). There are many possible challenges that may be encountered during the cryopreservation process both cooling step the biological material and warming it back to physiological temperatures. The main aim of the cryopreservation processes is to reach low temperatures without causing any damage caused by the formation of ice crystals during freezing step (Sağırkaya and Bağış 2003).

At sufficiently low temperatures, any enzymatic or chemical activity that potentially damage the biological material is effectively halted. Since the increase of the ionic composition after formation of the ice in dilute aqueous solutions such as growth media, the negative effects occur on cell viability and functions by increasing osmotic pressure. Therefore, to minimize all negative effects during the freezing and thawing steps, utilization of the CPAs is especially preferred (Sambu, 2015). During cryopreservation, factors such as solution effect, extracellular and intracellular ice formation, and dehydration can damage cells. The important negative effects on the cells, which occur during the cryopreservation process, are mainly extracellular and/ or intracellular ice crystals formation. One of the harmful effects during slowly cooling is the migration of the water out of the cell, in which extremely cellular dehydration occurs. As a result of this, excessive extracellular ice formation occurs, and cell membranes are mechanically damaged or cells are exposed to stress. While some tissues can tolerate extracellular ice formation, grow out of the intracellular ice crystals can be fatal for the most cells. Many of these harmful effects can be reduced with cryoprotectants (Mazur, 1984).

Controlled slow freezing and vitrification methods are used to prevent cryopreservation damages. One of the key success factors of the cryopreservation process is that cooling must be performed at a controlled rate. In this way, it is possible to optimize the harmful effects that will arise from the solution and ice crystals. If the cooling rate is too high or low, the cell viability ratio is quite low. Whereas the application of gradually cooling, extracellular ice is firstly formed due to moving the intracellular water towards outward. This approach is preferred in cryopreservation because it significantly reduces the potential for intracellular ice formation (Benson 2004).

In this way, the reasonable amount of water is removed to prevent the cell to damage. Consequently, ice formation is inhibited because of the amount of remaining water is very low. Also, the cytoplasm is probably to become glassy. In general, controlled slow freezing methods involve a series of treatments. These treatments consist of three main steps as follows: Precultured or conditioned and then cryopreserved of the samples (Benson, 2004). In general, it is preferred to use one of the permeable and non-permeable cryoprotectants or to use by mixing them in certain proportions. For example, DMSO as permeable CPA can be combined with sugar or polyethylene glycol as nonpermeable CPAs. Afterwards, a specific cooling rate (-0.1 to 5 °C min^{-1}) is applied to samples, often referred to as a "ramp". This process, which also is called two-stage cooling or freezing, continues until an intermediate transfer temperature of -35 to -40 °C. Samples can be immersed in liquid nitrogen at this point. When the cooling rate is slow, alcohol baths placed in the freezer at -20 or -80 °C or units containing alcohol mixtures (methanol-isopropanol) are used (Benson, 2004).

Vitrification is the "glassy state" of a solid or liquid materials, in which material is amorphous, extremely viscous and lacks organized structure but maintains their mechanical, physical and molecular properties. Since the vitrification process in biotechnology is a process performed by high-speed cooling of the cells in liquid nitrogen, also ice crystals cannot form. Therefore, the harmful effects of ice crystal formation which occur in conventional slow freezing cryopreservation methods, on the cells are not also observed. Vitrification is preferred an alternative to traditional freezing methods in particular many biotechnology applications because of a simple and less expensive process. There are five steps in the vitrification and thawing process for biological materials, as follows: (1) soaking of the sample in a solution containing cryoprotectant; (2) equilibration of the sample in a concentrated solution, in which the sample vitrifies by dehydration; (3) then immersion in liquid nitrogen; (4) at the end of the predetermined time, heating of the sample; (5) removal of the cryoprotectant from the sample (FAO, 2012). Nevertheless, cryopreservation can be used for the successful cryopreservation of many biological materials such as embryos, ovarian tissue, sperm, platelets, stem cells, and plant seeds (Jang, 2017).

We can divide cryoprotectant substances into two main classes according to their ability to penetrate the cell membrane: cryoprotectants that can penetrate the cell (membrane-permeable) and those (nonpermeable) that cannot. Membrane-permeable cryoprotectants have low molecular weight and include DMSO glycerol, ethylene glycol, propylene glycol and some other alcohols. Before freezing, due to the osmotic pressure difference, the liquid inside the cell is replaced by cryoprotectant substances, thus minimizing changes in cell volume. As a result of this, the damage to the cells during the freezing could be at low level because the minimizing of ice crystals inside the cell. In addition, cryoprotectants protect the cells during the period when intense dehydration occurs when the cells are surrounded by high concentrations of salt. Nonpermeable cryoprotectants are classified in two groups: Low and high molecular weight. Low molecular weight ones are glucose, sucrose, some other sugars and high molecular weight ones are polyvinyl alcohol, polyvinyl pyrrolidone and some other polymers. Low molecular weight ones have important effect by minimizing the formation of ice crystals by dehydrating the cells before cooling. Whereas high molecular weight ones do not exhibit a significant or noticeable harmful effects on the change in the shape and size of ice crystals. As mentioned before, incubation of the cells in the solution containing the CPAs to ensure that they reach an equilibrium intracellular concentration is both one of the important steps of freezing and the success criteria of freezing. Although the protective effects of cryoprotectants vary depending on the cell type, almost all of them exhibit similar effects when used at the same concentration. For example, the highest success ratios in freezing human embryos and blastocysts are achieved when propanediol and glycerol are used, respectively. Existing membrane-permeable cryoprotectants are toxic at effective concentrations due to the high osmotic pressure they cause. They can affect the differentiation of many cell types and must be rapidly removed from the medium at the post-thaw stage. Since even the most used cryoprotectants have some toxic effects, new cryoprotectant substances are being investigated. In addition, some non-toxic proteins and synthetic polymers have cryoprotective properties to some extent. However, nonpermeable cryoprotectants exhibit poor cryoprotective properties, therefore they are generally not preferred to be used alone due to their inadequate (Fahy, 2014).

Usage DMSO and glycerol as CPAs together with fetal bovine serum (FBS) or albumin as proteins is well-known application in the conventional cryopreservation procedure. In general, to use a 10% DMSO solution with FBS is the most efficient cryopreservation procedure to preserve the cells. The main reasons of adding FBS are to protect both the structural integrity of the cell membrane and cell viability. However,

infections can occur by cross contamination depending on the proteins (FBS or others) obtained from animals. Proteins should not be used without purification. Due to the disadvantages associated with traditional cryoprotective agents (CPAs) such as DMSO, glycol derivatives, and protein-based substances including their toxicity and potential to disrupt cell membrane integrity researchers have increasingly focused on developing new CPAs that are less toxic or non-toxic, and that better preserve cellular structures and functions during the freezing and thawing processes (Agca, 1994; Palasz and Mapletoft, 1996).

Polyampholytes: Structure, history, synthesis and cryoprotective properties

Polyampholytes are an important class of polymers consist of positively and negatively charged monomer subunits. Since polyampholytes contain both anionic and cationic functional groups in their structures, the strengths of these functional groups (i.e., both weak anionic and cationic, weak anionic and strong cationic, strong anionic and weak cationic, and both strong anionic and cationic) have an important effect on their general characteristics. The differences of these functional groups cause to change the net charge of a polyampholyte in aqueous solution by varying the pH value of the medium. When the numbers of positive and negative charges on the polyampholyte molecule are equal depending on pH, net charge of polyampholyte is zero. This specific pH value is called isoelectric point (IEP). Polyampholytes depending on their pH sensitivity characteristics exhibit polyelectrolyte-like behavior. This situation makes polyampholytes remarkable particularly for biotechnology applications. Therefore, polyampholytes due to their water solubility and swelling ability are being investigated as synthetic protein and the other materials alternatives in a wide range of applications, including tissue engineering, cryopreservation, drug delivery and nonfouling materials applications (Stubbs et al., 2020a).

Polyampholytes can be synthesized in the synthetic copolymer structure by using selected monomers, and some natural polymers are also in the polyampholyte structure. Examples of natural polyampholytes include proteins containing 20% amino acids, especially collagen. Synthetic polyampholytes have been developed more recently and are synthesized by using oppositely charged acrylamide or acrylic acid-based monomers such as methacrylic acid (MAA) with either 2-vinyl pyridine (Alfrey et al., 1952) or 2-dimethylaminoethyl methacrylate (2-DMAEMA) (Ehrlich and Doty, 1954) according to the conventional free radical addition copolymerization in the aqueous media (Ciferri and Kudaibergenov, 2007).

In the earlier studies in the literature were reported that polyampholytes, were synthesized by Turner for the first time in 1950, as a 'synthetic alternative to proteins' (Alfrey et al., 1950). It was indicated that a copolymer containing 2-vinylpyridine and MAA monomer units exhibited solubility and electrophoretic mobility characteristics like proteins (Alfrey et al., 1952). Subsequent research demonstrated that these materials have a polyion structures and can function as poly(anions) in alkaline solutions and poly(cations) in acidic solutions, while displaying a neutral charge near their IEP (Mumick et al., 1994; Li et al., 2010). Later advancements in living polymerization techniques facilitated the synthesis of the first polyampholyte blocks in the early 1970s (Kamachi et al., 1972). This involved the copolymerization of 2-vinyl pyridine with various methacrylate monomers by anionic addition polymerization mechanism. However, anionic polymerization presented significant practical challenges, limiting the synthesis of these materials to specialized laboratories. Since then, controlled radical techniques, including group transfer polymerization (GTP) (Patrickios et al., 1994), nitroxide-mediated polymerization (Gabaston et al., 1999), and reversible addition-fragmentation chain transfer polymerization (RAFT) (Colby et al., 2011), have been employed. More recently, Du Prez et al. (2017) to realize synthesis of polyampholytes with alternating copolymer structure suggested the ring-opening polymerization of an N-maleamic acid functionalized homocysteine thiolactone monomer. In this instance where direct polymerization of desired ampholytic units is not feasible, precursor materials are synthesized and subsequently modified post-polymerization. It was pointed out that this technique has been used to synthesize the polyampholytes from poly(amino acids) (Matsumura and Hyon, 2009) and maleic anhydride copolymers in purely alternating structure (Stubbs et al., 2017).

Matsumura and Hyon (2009) pioneered the investigation of polyampholytes as cryoprotectants. Synthesized polyampholytes with protein-like activities which prevent ice crystallization and contain a suitable ratio of amino and carboxyl groups without using other CPAs as an alternative to traditional cryoprotectants and obtained high-efficiency CPAs.

It was reported that when cells are frozen, cell death occurs due to intracellular ice formation (Mazur and Koshimoto, 2002). Membrane-permeable CPAs such as DMSO penetrate the cell and prevent intracellular ice formation. However, polyampholytes cannot penetrate the cell due to their high molecular weight and density and so they are nonpermeable.

Although the cryopreservation mechanism of polyampholytes is not fully elucidated, it is estimated that they exhibit cryoprotective properties by forming immediately soluble clusters in ice, capturing water and salt, acting as a buffer, preventing severe osmotic pressure changes and preventing crystal formation during freezing (Matsumura et al., 2013a). Polyampholytes also exhibit superior cryoprotective properties by preventing ice recrystallization during thawing (Mitchell et al., 2015). In recent studies, polyampholytes are preferred due to their low toxicity and good cryoprotective properties (Stubbs et al., 2020a).

Matsumura et al. (2013b), reported in another research that when the succinic anhydride modified ε-poly lysine was used as cryoprotectant without DMSO in the cryopreservation of stem cells, the toxic effect of this cryoprotectant was significantly low (Matsumura and Hyon, 2009). In another research presented in the literature noted that when the ratio of cationic and anionic groups in the polyampholyte molecular structure vary, their cryoprotective activity can vary. As result of this, it has been shown that a certain charge balance is necessary to obtain optimal cryoprotective activity of polyampholyte (Mitchell et al., 2014). Results indicated that ice recrystallization inhibition (IRI) activity, associated with cryoprotective ability, was maximized at a 1:1 charge ratio, with activity diminishing as one charge became dominant. Furthermore, the nature of the charge was critical, with zwitterionic monomers exhibiting no activity.

Rajan et al., (2013) synthesized copolymers of 2-DMAEMA and MMA containing different ratio of monomer units and it was demonstrated that 1:1 charge ratio is necessary to obtain optimal cryoprotective activity. It was indicated that notably when polyampholyte was hydrophobically modified by using monomers such as octyl or butyl methacrylate, both IRI activity of polyampholyte and cellular recovery post-thaw enhanced. It was observed that when poly (DMAEMA-MAA) was modified with butyl or octyl methacrylate, cellular recovery increased from approximately 60% to 90%. In further investigations, same characteristics of hydrophobically modified poly (DMAEMA-MAA) with poly(carboxymethyl betaine) (poly(CMB)) and poly(sulfobetaine) (poly(SPB)) were also investigated (Rajan and Matsumura, 2014). Hydrophobic modification significantly increases IRI activity, but long-term cell viability is poor. To increase cell viability, it was proposed addition of about 2% amount of DMSO to medium. Although poly(CMB) is structurally like poly(DMAEMA-MAA), it has weak IRI activity and not cryoprotective ability. These results presented in the literature showed that polymer structure and the functional groups are very important factors on its cryoprotective properties. Matsumura also synthesized polyampholyte hydrogel based cryoprotectants using click reactions and evaluated their efficacy in DMSO-free cryopreservation of mammalian cells (Jain et al., 2014).

Given the success of polyampholytes in suspension freezing, Matsumura explored their applicability in cryopreservation of cellular monolayers, which present greater freezing challenges (Armitage and Juss, 1996; Matsumura et al., 2016; Pasch et al.,1999). It was observed that when a cryoprotectant solution consists of ethylene glycol, COOH-εPLL, and sucrose during slow freezing, it has been stated that DMSO is not needed. COOH-εPLL has also been shown to be effective in cryopreservation of various cell types, including chondrocyte cell sheets (Maehara et al., 2013), mouse oocytes (Watanabe et al., 2013), pig (Kamoshita et al., 2017) and mouse (Shibao et al., 2014) embryos, rabbit sperm (Küçük at al., 2021) and human mesenchymal stem cells (Matsumura et al., 2013b).

Mitchell et al., (2015) reported that polyampholyte precursor in poly(methyl vinyl ether-alt-maleic anhydride) alternating copolymer structure was obtained in 1:1 charge ratios .This polyampholyte, combined with hydroxyethyl starch (HES), facilitated excellent blood cells recovery after a slow thawing technique.

The researchers realized many studies to elucidate of the mechanism of polyampholyte-mediated cryoprotection (Ahmed et al., 2016; Rajan et al., 2016). Polyampholyte-based nanocarrier complexes facilitated greater protein internalization than 10% DMSO, known to enhance membrane permeability (Ahmed et al., 2018). These findings indicated in the literature suggest that polyampholytes, while enhancing post-thaw cell viability, also interact with the membrane during freezing, promoting greater cellular uptake when incorporated into protein-nanocarrier complexes. Ahmed et al. (2019) utilized hydrophobic polyampholytes to enable gold nanoparticle internalization into cell-sized liposomes. These polyampholytes localized in the disordered phase of the membrane, facilitating greater particle internalization at low temperatures. Solid-state nuclear magnetic resonance (NMR) studies have elucidated the interaction mechanism of polyampholytes on ice and cells during cryopreservation, suggesting a vitrification tendency characterized by macroscopically limited mobility and increased solution viscosity. This indicates that potent interactions contribute to trapping water and salt within the matrix, reducing the damages

formed the intercellular ice formation and change of the osmotic pressure, which is beneficial for biomaterial cryopreservation (Matsumura et al., 2021). Furthermore, cell membrane protection occurs during polyampholyte adsorption on cell membranes during freezing. Beyond cell cryopreservation, polyampholytes enhance protein-nanocarrier complex adsorption onto cell membranes, it exhibits better performance than DMSO (Ahmed et al., 2018; Rajan et al., 2016). Hydrophobic polyampholytes with long alkyl side chains also protect proteins from harmful effects formed during freezing stages. This shows their ability to stabilize various proteins under different stress conditions (Dai et al., 2023). Grafted copolymers of polyvinyl alcohol (PVA) and succinylated ε-poly-L-lysine (PLLSA) inhibit osmotic stress and ice recrystallization, protecting proteins during long-term cryopreservation (Rajan et al., 2021). The grafting copolymers demonstrate higher efficiency in prolonged protein freezing compared to PVA and PLLSA alone. Subsequently, polyampholyte-based hydrogels containing PLLSA, PVA, and polyethylene glycol (PEG) were prepared, demonstrating protection in protein storage. These hydrogels prevent protein aggregation and denaturation, withstanding freezing stress for 15 freeze/thaw cycles between -80°C and 37°C. The native activity of insulin after cryopreservation at -80°C for 14 days was maintained at approximately 90%, due to protein entrapment within the polyampholyte hydrogels, preventing cryoinjuries (Rajan et al., 2023). To mitigate potential physiological toxicity, ester-containing biodegradable polyampholytes were synthesized, proving biocompatible for cell cryopreservation (Pesenti et al., 2022). Burkey et al., (2020) reported modified polyampholytes (PAGE-PA) derived from poly(methyl glycidyl sulfoxide) (PMGS), achieving near 80% post-thaw viability for fibroblasts and over 90% viability for 3T3 cells, through limiting freezing water and enhancing cellular dehydration after ice nucleation. However, PMGS toxicity, biodegradability and final product toxicity should be kept in mind. Ishibe et al., (2022) pointed out that DMSO-like macromolecular CPAs did not consistently outperform corresponding unoxidized sulfur-containing polymers, and uncontrollable overoxidation during preparation could result in excessive cellular cytotoxicity. Polyampholytes derived from modified poly(methyl vinyl ether-alt-maleic anhydride) as well-defined macromolecular CPAs exhibited definite ice recrystallization inhibition (IRI) activity and optimal alternating cationic/anionic charges for maximum activity and cryopreservation efficiency (Stubbs et al., 2020b). This alternating copolymer inhibited 50% ice recrystallization at 20 mg mL^{-1}, with minimal. A mixed CPA of polyampholytes (20 mg mL^{-1}) and hydroxyethyl starch (HES) protected ovine red blood cells (RBCs), achieving over 60% cell recovery at -196°C for 5 days (Mitchell et al., 2015). Due to high viscosity, obtaining higher concentration polyampholyte solutions is one of the challenges. The required dose was lower than that for stem cell cryopreservation using poly-L-lysine-based polyampholytes. Subsequently, a photochemical high-throughput discovery platform was developed to identify polyampholyte terpolymer CPAs via RAFT polymerization (Stubbs et al., 2020b). To replace traditional small molecule glycerol in RBC cryopreservation, mixed CPAs of polyampholytes, DMSO, and trehalose were developed to protect sheep RBCs. It was observed that these components provided distinct benefits for RBC cryopreservation. Optimal CPAs yielded up to 97% RBC recovery, maintaining structural and functional integrity. It was stated that only minor cell morphology shrinkage occurred in this cryopreservation, which could be optimized through rewarming and washing processes (Murray et al., 2021). Nevertheless, it was also reported that obtaining cryopreserved cell monolayers is challenging, due to difficulties in cell adsorption onto tissues. Tomás, et al. (2022) proposed a reproducible cryopreservation approach using macromolecular polyampholyte CPAs for common cell lines (A549, HepG2, and Caco-2), maintaining cellular abilities and functions. Confocal microscopy images indicated minimal injury and preserved cell membrane integrity post-cryopreservation, due to polyampholyte-promoted cellular dehydration and reduced intracellular ice formation. Cryopreservation of HepG2 cells using mixed CPAs of DMSO, polyampholytes, ice nucleators (cholesterol), and Hornbeam pollen washing water, achieved approximately 75% recovery of primary hepatocytes after 24 hours post-thaw (Dallman et al., 2023). This simplified mixed CPA approach improved efficiency and commercial viability. Bissoyi et al. (2023) used poly(methyl vinyl ether-alt-maleic anhydride)-derived polyampholyte modified with dimethyl amino ethanol to protect liver-cell spheroids. Compared to DMSO-only CPAs, polyampholyte addition increased post-thaw recovery, maintaining cytoskeleton and cell membrane integrity. Macromolecular CPAs removal is easy and time-saving due to extracellular localization, added functionality and biocompatibility (Marton et al., 2023a). Polyampholytes are thus considered indispensable additives for enhancing post-thaw recovery. It was stated that poly(acrylic acid) (PAA) addition, as anionic water-soluble polymers, prevented phage infection in bacterial growth, a virustatic rather

than virucidal mechanism, while poly(methacrylic acid) was less active (Marton et al., 2023b). This provides insights for phage cryopreservation. Liu et al., (2019) developed a DMSO-free chondrocyte cryopreservation method. Mixed CPAs of betaine and poly (carboxybetaine methacrylate) (PCBMA) and poly (sulfobetaine methacrylate) (PSBMA) zwitterionic polymers were biocompatible, achieving over 90% post -thaw viability efficiency after 4 hours incubation at 37° C. It is thought that this situation mainly caused by enhancing betaine absorption because of decreasing the electrostatic interaction between intrachain ionic pairs in the polymer backbone and increasing extracellular permeability. PCBMA was also shown to have IRI activity, unlike pure water (Chen et al., 2023).

Conclusion

Cryopreservation is a vital technique for preserving biological materials such as especially mammalian cells for various applications. However, ice crystal formation during freezing and thawing remains a significant challenge. Polyampholytes and polyzwitterions have emerged as promising cryoprotective agents due to their unique properties, including their ability to interact with cell membranes, prevent ice crystal formation, and reduce osmotic stress.

This review has highlighted the history of cryopreservation, fundamental concepts, and the synthesis and cryoprotective properties of polyampholytes. The studies discussed demonstrate the potential of polyampholytes as effective CPAs for various cell types and applications. However, further research is needed to optimize their cryoprotective efficacy and minimize potential toxicity.

By addressing the research directions outlined in the "Future Perspectives" section, we can further advance the field of cryopreservation and develop more effective and safe methods for preserving biological materials.

Future Perspectives:

Despite the promising results obtained with polyampholytes and polyzwitterions in cryopreservation, several challenges remain. Future research should focus on:

Development of novel polyampholyte structures: Exploring different monomer combinations and new modification techniques to synthesize polyampholytes with improved cryoprotective properties.

Investigation of the mechanisms of polyampholyte-mediated cryoprotection: Elucidating the interactions between polyampholytes and cell membranes, as well as their effects on ice crystal formation and osmotic stress.

Evaluation of the biocompatibility and toxicity of polyampholytes: Conducting comprehensive in vitro and in vivo studies to assess the safety and efficacy of polyampholytes for clinical applications.

Development of standardized cryopreservation protocols: Establishing optimized protocols for different cell types and applications, including the use of polyampholytes as CPAs.

Investigation of the utilization of polyampholytes in combination with other CPAs: Investigating synergistic effects between polyampholytes and traditional CPAs to enhance cryoprotective efficacy.

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