# 博士論文

# Artificial zwitterions as designable cryoprotectants

# 設計可能な凍結保存剤としての人工双性イオン

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提出年月： 2024/03/22

# 内容

# 1. Significance of cryopreservation

## 1.1 Cell

### Cell

Cells are the smallest units that form individual animals and plants[1]. Cells are surrounded by a membrane structure that separates the cytoplasm from the outside world. They have internal organs that constantly perform life activities by carrying out metabolic pathways[2] such as the glycolysis system and the citric acid cycle, and expresses genetic information for self-renewal and replication[2, 3]. Living organisms are aggregates of cells with various functions, and carry out higher-order life activities through intercellular interactions. Therefore, cells are important research materials in research to understand and elucidate life phenomena.

Animal cells are an integral part of biomedical research. As models for human systems, researchers can use animal cell lines to investigate a wide range of disease mechanisms and evaluate new therapeutics in animal models before applying these findings to humans. Many animal cell lines are not only useful as disease models, but also essential for bioindustrial applications such as recombinant protein expression, virus production, pathogen detection, and toxicity screening. Furthermore, animal cell lines can provide new insights into the areas of developmental biology, intracellular signaling, and genetic evolution[4]. In addition, with the advent of induced pluripotent stem cell establishment technology, cell research has shifted to a new paradigm.

A research cell line is, in a broad sense, used for a cell population that has acquired certain stable properties after subculturing for some time. In a narrow sense, it is used for cell populations that have been immortalized and can be cultured for a long period[1]. Immortalization is used as having acquired the ability to continue proliferating semi-permanently in a test tube.

### Cell culture

When using cells as research materials, cells are taken out from individual animals or plants and used. However, when trying to study higher-order biological activities in cells, it is essential to observe cells in a living state. Thus, techniques have been established to keep cells alive in vitro. This is the beginning of cell culture[1]. Living organisms are not static, but evolve through the accumulation of genetic mutations. Of course, genetic mutations inevitably accumulate in cells cultured in vitro. ES cells and iPS cells require more careful management than normal cells to maintain their cell characteristics. Specifically, quality control such as periodic analysis of undifferentiated markers and confirmation of teratoma formation after transplantation into immunodeficient mice is required.

### Cell death

Factors that cause cell death include environmental conditions such as temperature and ion concentration, immune abnormalities, viral infections, inflammation, radiation, and chemical

substances. Although there are many factors, cell death itself is thought to occur by two mechanisms, apoptosis and necrosis. There are many observable morphological and biochemical differences between the two types of cell death.

When cells proliferate indefinitely, a tumor, which is a mass of cells, is formed. In order to prevent this tumorigenesis from occurring, cell growth and proliferation are regulated in vivo through a series of morphological changes called apoptosis. Therefore, apoptosis is also called "programmed cell death". It prevents the formation of abnormal tissues by cells committing suicide, and plays an important role in each stage of normal tissue homeostasis and defense, aging, and individual death. In apoptotic cells, the nucleus first aggregates and DNA fragments. Shortly thereafter, the cells retire, fragment, and release membrane-enclosed apoptotic bodies. Apoptotic bodies are normally phagocytosed by other cells (Fig.1-1). In this way, intracellular substances are released into the extracellular environment without adversely affecting the surrounding cells.

In contrast to apoptosis, cell death occurs through a process called necrosis when cells are physically or chemically injured (Fig. 1-2). Necrosis generally begins with an imbalance in the homeostatic capacity of the cell, leading to an influx of water and extracellular ions into the cell. Cellular organelles, especially mitochondria, and the entire cell swell, causing cytolysis in which the cell bursts. Ultimately, the plasma membrane is disrupted, releasing the cytoplasmic contents, including enzymes of lysosomal origin, outside the cell. As a result, there is a possibility that the released intracellular substances may damage surrounding cells or cause inflammation.

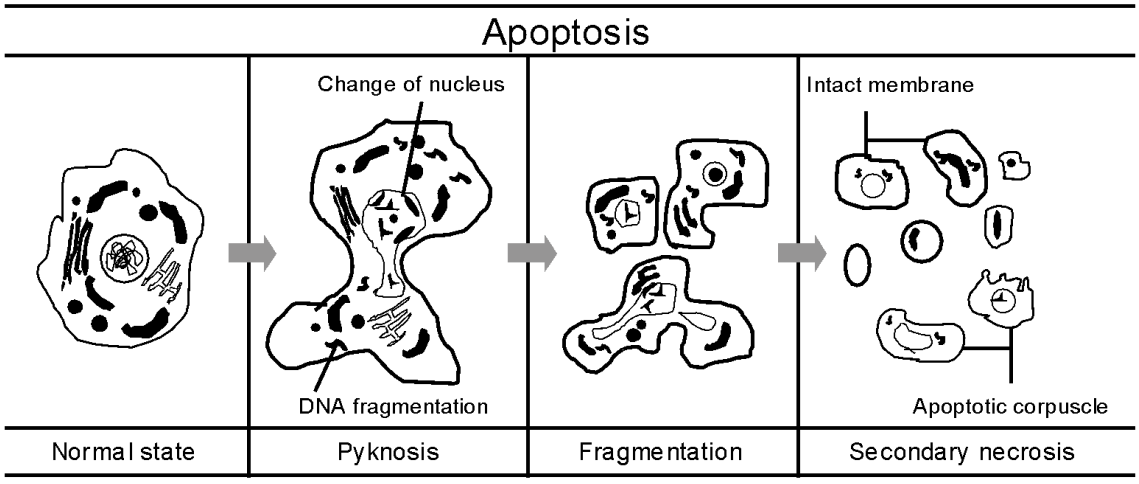



Fig. 1-1 Scheme of apoptosis process

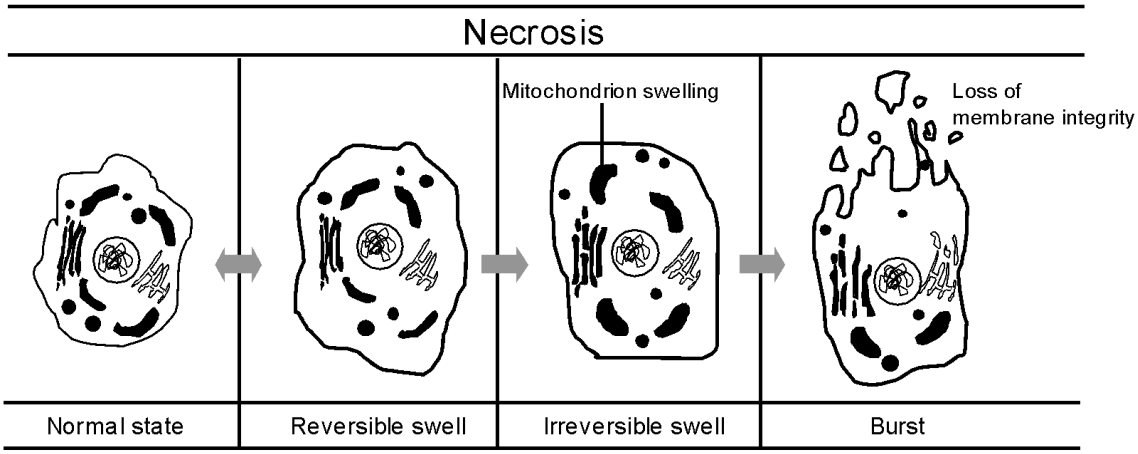



Fig. 1-2 Scheme of necrosis process

## 1.2 Cryopreservation

### Significance of cryopreservation

Continuing the subculturing alone increases the probability of mutation and may change various characteristics of the cells. Therefore, storage at ultra-low temperatures is essential for long-term stable use of cells[5, 6]. At present, more than 3,600 cells are cryopreserved in the ATCC bank[4]. In the five years from 2010 to 2014, 34,753 cases of oocyte cryopreservation for fertility treatment were reported in 17 European countries[7]. Usually, cells are suspended in medium containing 10–20% serum supplemented with 10% dimethyl sulfoxide (DMSO) and cryopreserved in a -80 °C freezer or liquid nitrogen. It can be stored for one to several years in this state. Frozen cells are thawed at 37 °C, washed and cultured to begin proliferation. Preservation in liquid nitrogen tanks (liquid phase -196 °C, gas phase -165 °C) can be stably preserved with almost no change in cell properties, so it is used to preserve cells derived from rare specimens, long-lived cells, embryonic cells, and various animal cells. Therefore, cryopreservation and thawing of cultured cells are one of the most basic techniques for research involving cells[8].

### Cryopreservation type

Freezing the cell suspension kills most of the cells. This biggest problem is the physical destruction of cells due to the formation of ice crystals[9]. In order to suppress the ice crystal formation, the rapid freezing method and the slow freezing method using a freezing medium were developed (Fig. 1-3).

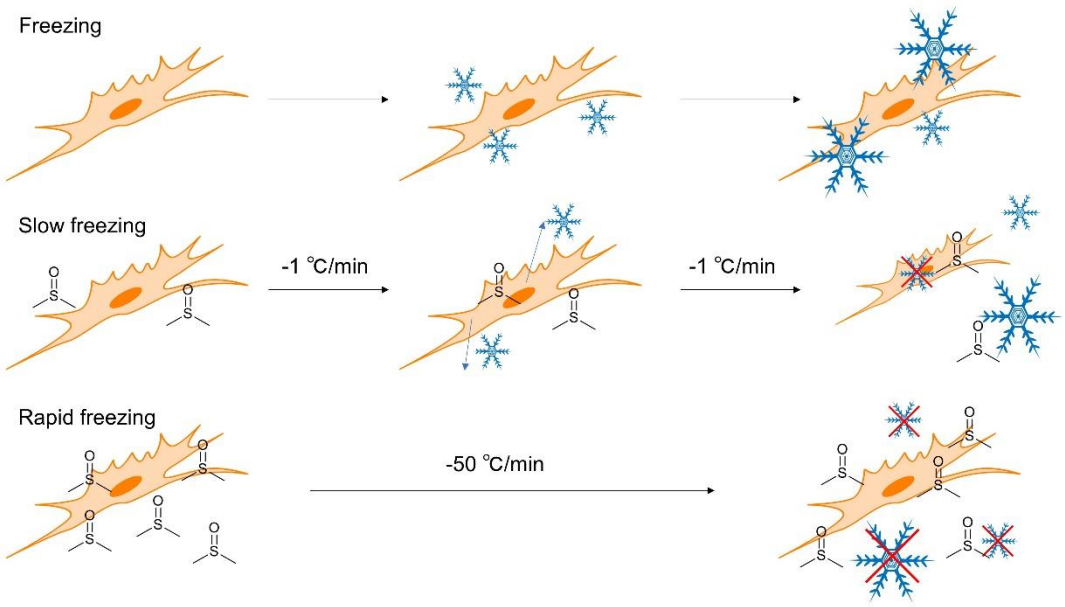



Fig. 1-3 Schematic diagram of slow freezing method and rapid freezing method.

**・Slow freezing**

In the slow freezing method, the cells are immersed in a freezing medium containing a relatively low concentration of cryoprotectant (around 5-20%) and slowly cooled at a rate of about 1 °C/min to freeze the cells. Rapid freezing causes the water inside the cells to become ice, damaging and killing the cells. Slow freezing causes gradual crystallization of extracellular water, which causes osmotic pressure difference between the inside and outside of the cells, thereby gradually dehydrating the inside of the cells. Eventually, as ice crystal formation proceeds further outside the cell, the freezing medium inside and around the cell is sufficiently concentrated, and a vitrification state is reached in which ice crystals are not formed even at ultra-low temperatures in liquid nitrogen. A vitrification state is stable because it does not have a specific molecular arrangement and the movement of molecules is suppressed. In this case, the viscosity of the freezing medium becomes extremely high. In the vitrification state, large hard ice crystals do not form, so no disruption of the cellular structure occurs. Freezing media use osmotic pressure differentials to force water out of cells and instead enter cells to prevent ice build-up. When cryopreserving cells, it is common to use a program freezer, a commercially available special container (®Mr. Frosty), a Styrofoam container, etc. to freeze gradually while suppressing the growth of ice. This slow freezing method is used for animal cells in general.

**・Rapid freezing**

The rapid freezing method involves immersing the cells in a vitrification freezing medium and quickly immersing them in liquid nitrogen to quickly freeze them. The vitrifying freezing medium was

developed as a freezing medium that does not form ice crystals even when immersed in liquid nitrogen. It generally contains high concentrations of antifreeze agents such as glycerol, ethylene glycol, propylene glycol. It has a high osmotic pressure in freezing medium and is highly toxic to cells. Therefore, in order to minimize the use of liquids that are toxic to cells, a quick and accurate freezing operation is required. This rapid freezing method is widely used in animal experiments such as mice and rabbits, cryopreservation of early embryos and fertilized eggs of livestock such as cows and pigs, and cryopreservation of eggs and fertilized eggs used for human infertility treatment.

**Limitations and possibilities of current cryopreservation**

The rapid freezing method freezes rapidly by immersing it in liquid nitrogen, which causes the freezing medium to vitrify and prevents ice crystal formation, thereby minimizing damage to the cells. However, a high concentration of cryoprotectant is required to achieve a vitrified state. On the other hand, the slow freezing method is a method for suppressing ice crystal formation by slowly freezing a freezing medium and dehydrating the inside of cells. However, this method has the problem that it is immersed in a freezing medium for a long time, and ice crystal formation is not completely suppressed. The slow freezing method is widely used as a technique for cryopreservation, but depending on the cell type, the cell viability after thawing may be extremely low. Pluripotent stem cells such as human ES cells, human iPS cells, and monkey-derived pluripotent stem cells such as cynomolgus monkeys are known to have a very low cell viability after freezing and thawing by the slow freezing method. As a means to solve this problem, a rapid freezing method has been devised, and various improvements are underway, such as the development of a vitrification freezing medium that is simpler, more efficient, and has lower toxicity. However, the freezing medium used in the rapid freezing method generally contains high concentrations of cryoprotectants such as glycerol, ethylene glycol, propylene glycol. It has a high osmotic pressure in freezing medium and is highly toxic to cells. Therefore, in order to minimize the use of liquids that are toxic to cells, a quick and accurate freezing operation is required.

Slow freezing limits the number of cells that can be frozen, and rapid freezing has the problem that the cryopreservation agent is toxic and operation is difficult. Therefore, both cryopreservation methods have drawbacks, and no method or freezing medium will cryopreserve all cells.

## 1.3 Cryoprotectant agents

**Role of cryoprotectants**

Since cells contain a large amount of water, when cultured cells are frozen in a culture solution, the water inside and outside the cells crystallizes. This ice crystal physically damages cell structures such as cell membranes and intracellular organelles, and the cells cannot survive after thawing. Therefore, for cryopreservation of cells, it is particularly important to prevent ice crystal formation in

and near cells, and to prevent physical damage to cell tissues[5].

Here, a cryoprotectant that has the effect of suppressing ice crystal formation is required. When cells are frozen with the addition of cryoprotectant, the extracellular water is frozen and the solutes excluded from the ice crystals are concentrated in a process called cryoconcentration[10]. This is one mechanism for dehydrating cells and avoiding cryo-injury by increasing their concentration in the freezing medium around the cells, preventing lethal intracellular ice crystal formation. This can protect the cells from ice crystals.

The first breakthrough in the field of cryopreservation was achieved by Polge *et al.* in 1949, when they reported that the addition of glycerol enables fowl sperm to be cryopreserved[11]. DMSO was subsequently found to efficiently cryopreserve red blood cells[12], which caught the attention of cryobiologists worldwide and became the most preferred cryoprotectant, mainly because of its ability to penetrate cells. Although glycerol was eventually found to only exhibit weak cryoprotective properties, and DMSO is cytotoxic to cells[13-15], these freezing media were inevitably used because of a lack of efficient alternatives; hence, the development of more efficient freezing media is of paramount importance.

A typical composition of freezing medium is 10% DMSO, 20% fetal bovine serum (FBS), 70% culture medium (D-MEM). However, the composition of the commercial on sale has not been disclosed. DMSO in the composition can enter the cell and interact with water molecules to inhibit ice crystal formation inside and outside the cell. FBS also inhibits ice crystal formation by interacting with water molecules.

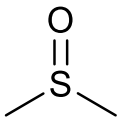
There are two types of cryoprotectants: cell-permeant and non-cell-permeant.

**Cell-permeable cryoprotectants**

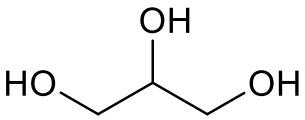
DMSO (Fig. 1-4), a common cryoprotectant, is cell permeable[12, 16], and DMSO inhibits ice crystal formation by interacting with water molecules intra- and extracellularly[17]. However, DMSO is known to permeate cells and disrupt intracellular functions, and in the worst cases, induce cell apoptosis and misdifferentiation[13-15, 18].

DMSO is an organic compound and a most commonly used non-aqueous solvent that is freely miscible with water and dissolves many organic compounds and inorganic salts[19]. It is classified as Class 3 (non-toxic) by the US Food and Drug Administration (FDA) and the International Harmonization Committee (ICH)[20, 21]. However, DMSO is toxic. It can damage cells and severely affect their behavior. DMSO is a cell-permeable reagent known to bind to a variety of cytoplasmic or nuclear proteins[16], directly disrupting their function[18]. These intracellular modifications indirectly alter cellular behavior[22, 23], causing endoplasmic reticulum stress that induces mitochondrial depolarization/dysfunction and apoptosis[24]. DMSO is therefore essential but not a universal solvent in

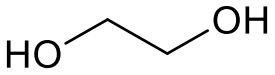
life sciences.




A structure of DMSO.




A structure of glycerol.




A structure of ethylene glycol.

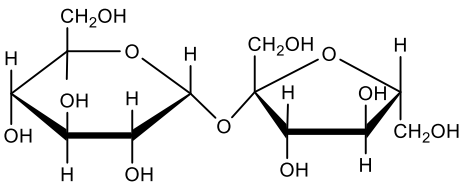
Fig. 1-4 Structures of cell-permeable cryoprotectant.

**Non-cell-permeant cryoprotectants**

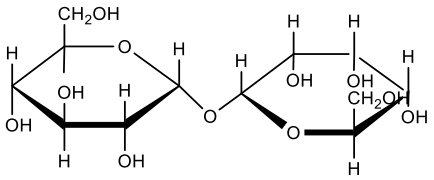
Sucrose and trehalose are known as non-cell-permeating cryoprotectants (Fig 1-5), and since they do not permeate cells, they are basically less toxic to cells[25]. Although these suppress extracellular ice crystal formation by interacting with extracellular water, they have the disadvantage that they cannot directly suppress intracellular ice crystal formation. Therefore, it is necessary to increase the osmotic pressure to 2 to 3 times the isotonic pressure to dehydrate the cells and indirectly suppress intracellular ice crystal formation. However, placing cells in a hypertonic freezing medium and rapidly dehydrating them can still cause significant damage to the cells[26]. Therefore, it is expected to be an effective freezing medium that exhibits the highest cryopreservation effect under isotonic conditions and is non-cell-permeant.

A previous study with hydroxyethyl starch (HES), a non-cell-permeable cryoprotectant, suggested that it attracts and absorbs water from outside the membrane, thereby reducing the viscosity, increasing the rate of dehydration, and impeding intracellular ice crystal formation[27, 28]. However, HES is weakly cryoprotective and is not suitable for all cell types. In contrast, polyampholytes are efficient cryoprotectants that can be used for various cell types; hence, they should be better able to control dehydration from outside the membrane by controlling osmotic stress during freeze concentration.

In nature, intrinsically disordered proteins (IDPs), such as late embryogenesis abundant (LEA) proteins, protect organisms from dehydration stress caused by desiccation or osmotic stress at low temperatures[29, 30-32]. Although IDPs have been speculated to protect desiccation-sensitive macromolecules by vitrification, the mechanism remains unclear. Since IDPs are charged molecules, like polyampholytes, they are good models for elucidating the mechanism behind desiccation tolerance. To the best of our knowledge, cryopreservation and desiccation have not been quantitatively analyzed from the viewpoint of dehydration control through the molecular structure.




A structure of sucrose.




A structure of trehalose.

Fig. 1-5 Structures of non-cell-permeable cryoprotectant.

## 1.4 Ionic liquids

Ionic liquids are defined as “liquids consisting only of ions, which have a melting point near room temperature”[33]. In general, a salt is a substance consisting only of an anion and a cation, and common salt (NaCl) is a typical example. Inorganic salts such as NaCl are composed of small ions, and the electrostatic interactions between ions are so strong that they must be heated above 800 °C to become liquid. Ionic liquids have a lower melting point and exist in a liquid state even at room temperature by replacing the ions that make up the salt with large-sized organic ions. For this reason, it is also called a third liquid that is neither water nor an organic compound.

Ionic liquids were synthesized by wilkes *et al.* Interest in ionic liquids has exploded because they can be used not only as electrical media but also as media for chemical reactions and separations. The attractiveness of ionic liquids is many. For example, low volatility, high thermal stability, nonflammability, high ionic conductivity, unique ability to dissolve substances, wide potential window, and the like. The most attractive thing is that it is possible to synthesize thousands or tens of thousands of ionic liquids simply by changing the substituents and combinations because it is possible to design physical properties and functions since the majority of constituent ions are organic ions. For this reason, ionic liquids are also called “designer liquids”[34]. Ionic liquids are attracting attention not only for their use as environmentally friendly solvents, but also for their ability to freely adjust their structures and physical properties. In addition, it has various properties in terms of physical properties, such as a unique phenomenon completely different from that of water or organic solvents, and the existence of a domain structure with hierarchy.

The ionic liquid can be designed with cations and anions according to the intended use. In other words, it can be said that it is a rare solvent whose interaction can be controlled as desired by significantly changing its structure. For example, Ohno *et al.* combined the two properties of high polarity and hydrophobicity, which are usually incompatible, by making the anion highly polar and the cation hydrophobic[35, 36]. As a result, cellulose in hydrophobic ionic liquids can be dissolved, which cannot be achieved with molecular liquids. Due to the vast number of combinations of cations and anions, it is difficult to design ionic liquids with targeted properties. Therefore, it is important to know the basic physical properties of ionic liquids.

In this paper, the properties of ionic liquids, especially their thermal properties and ionicity, are discussed.

### ・**Thermal properties**

Considering the application of ionic liquids to the real world, it is desirable that they maintain their fluidity without causing crystallization without heating. However, among the huge variety of

salts, there are few ionic liquids that can remain liquid at room temperature. To achieve this, we need to understand the melting point of ionic liquids.

The melting point of a salt strongly depends on the structure of its constituent ions. For example, the inorganic salt NaCl has a melting point of 801 °C, while [$C_2$mim]Cl has a melting point of 89 °C. This two things differences are the ionic radius of the cation and the presence or absence of charge delocalization. The high melting point of many salts is due to lattice energy acting between ions. As a result of lowering the lattice energy by increasing the ionic radius and delocalizing the charge, the melting point can be adjusted to near room temperature. In order to prevent crystallization, it is also effective to make the ionic structure asymmetric and control the van der Waals force.

**・Electrical properties**

Ionic liquids can be divided into four groups based on their Lewis acidity and basicity[37] (Fig. 1-6). Type I uses strong ions for both Lewis acidity and basicity. Type I tends to be a salt with a high melting point, like NaCl, and never an ionic liquid. Type IV uses weak ions that for both Lewis acidity and basicity. Unlike type I, type IV can become an ionized ionic liquid even at room temperature. This is because the type IV ions behave like "solvated ions". In other words, although the interaction between the ion pairs is comparable to that of covalent bonds, they are in an ionized state, so typical properties of ionic liquids such as high thermal stability and flame retardancy appear.

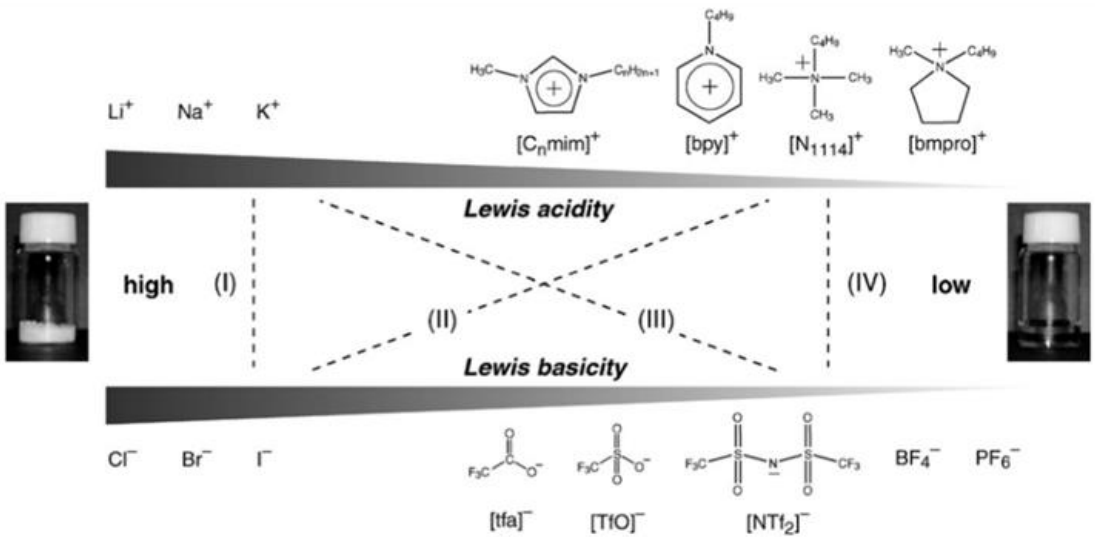



Fig. 1-6 A salts composed to cations and anions, respectively.

**Synthesis of ionic liquids**

Synthesis methods for ionic liquids include anion exchange method, acid ester method, neutralization method, and the like. Some synthetic methods will be described.

**・Quaternization reaction**

The quaternization reaction is a method for synthesizing ionic liquids by quaternizing tertiary amines and phosphines. Due to its reaction characteristics, it is used to synthesize ionic liquids with halide anions. These alkylamines and phosphines are excellent nucleophiles in SN2 reactions, and SN2 alkylation proceeds by reacting alkylamines and phosphines with alkyl halides. It is possible to synthesize ammonium salts and phosphonium salts by quaternizing not only imidazolium salts but also tertiary amines and tertiary phosphines. The alkyl halides used in these reactions include Cl-, Br-, and I-isomers, and the reaction conditions such as time and temperature required for the reaction differ, since they differ in reactivity. For example, chloroalkanes are quaternized by reacting at 80 °C for a few days. On the other hand, bromoalkanes can be reacted by heating for about 24 hours. Also, since the reaction is based on SN2, the reaction rate is increased by using an aprotic solvent such as acetonitrile or tetrahydrofuran. Furthermore, the halide salt synthesized in this way can be subjected to anion exchange by using an ion exchange resin, and therefore is used for the synthesis of an ionic liquid by the anion exchange method described below.

**・Anion exchange**

Synthesis of ionic liquids using the anion exchange method can be mainly divided into two processes. The first process is the quaternization reaction step of synthesizing the target cation structure by quaternizing a compound such as a tertiary amine that becomes a cation structure, as described above. The second process is a two-step anion exchange step that exchanges the halide anion for the desired anion. Since the first process, the synthesis of the target cation by the quaternization reaction, has already been described, only the anion exchange process will be described here. Various methods have been proposed for the anion exchange reaction depending on the desired anion species. Here, a method for synthesizing ionic liquids based on imidazolium cations by the anion exchange method is described.

According to the HSAB (Hard and Soft Acids and Bases) rule, anion exchange occurs between the lithium salt containing TFSI and $CF_3SO_3$, which show relatively soft properties, and the imidazolium salt, which contains halide ions showing hard properties. For purification, the TFSI salt is separated into the dichloromethane phase and the lithium salt into the aqueous phase by separating the dichloromethane and water, so the desired ionic liquid can be easily obtained (Fig. 1- 8). However, it has the disadvantage that the range of application is limited due to the limited design of lithium ions required.

J. Golding *et al.* have proposed a method using an ion-exchange resin as a simpler method for producing an ionic liquid. In this method, by passing a salt containing the target cation through a column packed with an anion exchange resin, the anion is exchanged and the OH form can be obtained. After the exchange, anion exchange can be performed by neutralizing with a target acid or hydrolyzing

with an acid ester.

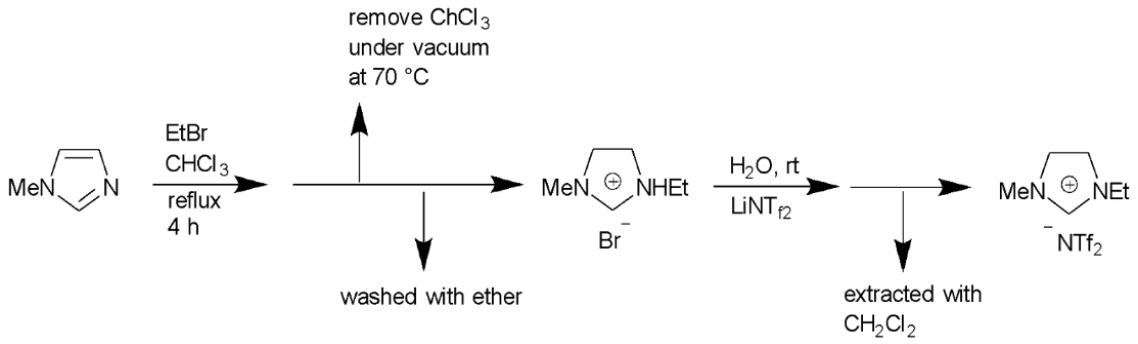



Fig. 1-8 A simple synthesis method of ionic liquid.

**・Transesterification**

Most ionic liquids can be made using the quaternization reaction method and anion exchange described above. However, since these are multi-step reactions, transesterification is proposed as a further costly and rapid synthesis. In this method, the Lewis base is quaternized and positively charged, while the leaving group is negatively charged as a Lewis acid, resulting in overall salt formation. In this method, the structure of the ether limits the design range and reactivity of the ions, thus limiting its applicability to a wide range. However, it can be made in one step and with a high collection rate.

**Purification of ionic liquids**

Since ionic liquids are salts that are liquid at room temperature and are hardly volatile, it is difficult to produce highly pure ionic liquids by synthesis and purification methods such as those for ordinary chemical substances. This requires the use of special refinements to synthesize ionic liquids. Several purification methods are described here.

**・Separation operation**

Ionic liquids are difficult to dissolve in nonpolar solvents such as hexane and diethyl ether because cations and anions interact gently due to electrostatic interactions. On the other hand, starting materials such as imidazole, phosphine, and alkyl halides are soluble in such solvents. Therefore, by dropping the synthesized ionic liquid into such a poor solvent and stirring, only the starting material can be selectively removed. In many cases, the starting material and the product can be separated by separating two liquids with different solubilities. For example, in a reaction in which an excess amount of phosphine is added to the OH form containing a phosphonium cation, the ionic liquid is extracted into the acetonitrile phase and the phosphine is extracted into the hexane phase by performing a liquid separation operation using an acetonitrile/hexane two-phase system. However, since the solubility in solvents changes dramatically depending on the ion design, solubility tests of starting materials and

reactants must be performed in advance. Thus, ionic liquids can be easily purified by considering the solubility of starting materials and reactants and using an appropriate two-phase system.

**・Column chromatography**

A common method for purifying ionic liquids is column chromatography, in which alumina and silica are used as fillers. Alumina ($Al_2O_3$) has basic (pH10), neutral (pH7.5) and acidic (pH4) types of adsorbents in alumina. By selecting these alumina species, most organic compounds other than saturated aliphatic hydrocarbons can be adsorbed. Acidic alumina can be used for separation of amino acids and acidic peptides, neutral alumina can be used for lactones, esters, ketosteroids, and basic alumina can be used for highly polar compounds. Salts are also selectively adsorbed on silica because the oxygen atoms of silica have a partial negative charge. By passing the reaction solution through a normal phase column, the product ionic liquid is adsorbed on silica, and only the reactants can be selectively flowed. By increasing the polarity of the solvent, the adsorbed ionic liquid can also flow, and the ionic liquid can be easily purified.

**Applications of ionic liquids**

Applications of ionic liquids in various fields have been investigated. Ionic liquids are expected to have endless possibilities not only in structural properties but also in applications. Here, we will introduce some of them.

**・Electrolytes**

An electrochemical device consists of an ion-conducting electrolyte and two electrodes sandwiching it. Conventional electrolyte solutions are obtained by adding molecular solvents such as water and organic solvents to salts containing ions, and dissolving and ionizing them. Ionic liquids are room-temperature molten salts having a melting point near room temperature, and typical ones are ionized even in the absence of a solvent, and thus exhibit high ionic conductivity. Since ionic liquids move as ionized ions without the addition of molecular solvents, they can be used as electrolytes without molecular solvents. In addition, due to its characteristics such as flame retardancy, low volatility, wide liquid temperature range, high ion concentration, and high decomposition voltage, it is expected to be applied to electric devices such as lithium batteries, electric double layer capacitors, and fuel cells as a safe next-generation electrolyte.

In particular, the electric double layer capacitor (EDLC) is an example of the early practical application of ionic liquids to electric devices[33]. EDLC is a device that uses the generated electric double layer charge to store electricity in a polarized region such as activated carbon, which has a large surface area and does not cause an electrochemical reaction with the electrolyte solution. Although it has a lower energy density than a battery, it can be discharged with a large current and has

a long life, so it was developed as a power source for computer memory backup. The electrochemical behavior of the electrode-ionic liquid interface for utilizing ionic liquids as electrolytes in EDLC was reported by Koch *et al.* They showed that the capacitance of ionic liquids is somewhat smaller than that of common aqueous electrolyte solutions, but larger than that of organic electrolytes[38]. From this, it is expected that the use of ionic liquids as electrolytes will increase the capacity and the possibility of greater energy storage, and that higher performance will be achieved compared to conventional EDLC. On the other hand, the capacity decreases due to the decrease in ionic conductivity accompanying the increase in the viscosity of the ionic liquid at low temperatures and the crystallization at the melting point. This is considered the biggest problem in entrapping ionic liquids into electrolytes.

**・Extractants**

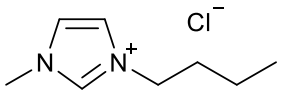
Ionic liquids are expected to be developed in various fields because they have characteristics that are significantly different from general molecular solvents, but this is not the only thing that deserves attention. By mixing ionic liquids with different properties, the physical properties can be changed continuously. Furthermore, when an ionic liquid and a molecular solvent are mixed, the ionic liquid alone may exhibit novel properties that have not been observed. Depending on the combination, an ionic liquid/molecular solvent two-phase system may be constructed. Previously, various liquid/liquid two-phase systems such as ionic liquid/alcohol system[39], ionic liquid/hydrocarbon mixed system[40], and ionic liquid/water mixed system[41, 42] have been reported. Of particular interest are hydrophobic ionic liquids that phase-separate from water. It is expected to be a new extraction solvent to replace hydrophobic organic solvents, and it has already been reported that highly hydrophobic organic compounds such as benzene derivatives can be extracted from the aqueous phase[41]. Such ionic liquids are also applied to the separation and extraction of metal ions[43] and proteins[44].

**・Biomass solvents**

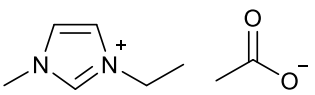
In recent years, the world has sought to find alternative energy sources to petroleum, which is thought to be depleted sooner or later. From the predictions, a society infer dependent on solar energy. On the other hand, it is becoming important to develop with awareness of "plant symbiosis," which can be seen as something that solar energy has changed. One of these developments is the conversion of biomass energy. Cellulose is the largest amount of non-edible biomass on earth. Cellulose is a polysaccharide whose basic unit is glucose, and since useful substances such as glucose can be produced from cellulose, it is attracting attention as a variety of energy sources. However, since cellulose forms multiple intramolecular and intermolecular covalent bonds, cellulose does not dissolve in water or commonly used polar organic solvents. It is well known that it is difficult to extract and dissolve using common solvents because it forms a polymer complex with cellulose, hemicellulose,

and lignin. For this reason, the effective use of biomass is emphasized but, the existing technology of processing under strong acid, high temperature, and high pressure requires a large amount of energy input, so it does not lead to substantial energy acquisition. Ionic liquids have attracted attention as new cellulose solvents.

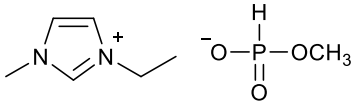
In 2002, Rogers *et al.* reported that 1,3-dialkylimidazolium chloride dissolves cellulose under heating[45]. However, chloride salts have a relatively high melting point and always require heating above 100 °C to handle them as liquids. In order to overcome these drawbacks, a low-viscosity chloride salt that is liquid at room temperature are obtained by designing an ionic liquid with a modified cation structure[46]. Modification of the ionic structure was made in order to further improve the physical properties. The key to finding solvent candidates for cellulose was to design ionic liquids that exhibit high hydrogen bond acceptability[47, 48]. Subsequent studies reported that ionic liquids with strong hydrogen bond acceptability, such as carboxylic acid-based ionic liquids and phosphate-based ionic liquids, dissolved cellulose (Fig. 1-9, 1-10). In recent years, there have been reports of attempts to treat cellulose-based biomass such as wood using ionic liquids[49]. Since these precedents have the potential to be epoch-making methods that can compensate for the shortcomings of conventional processing systems, the interest is rapidly spreading.



Chloride



Carboxylic acid



Phosphate type

Fig. 1-9 Structures of ionic liquid that dissolves cellulose.

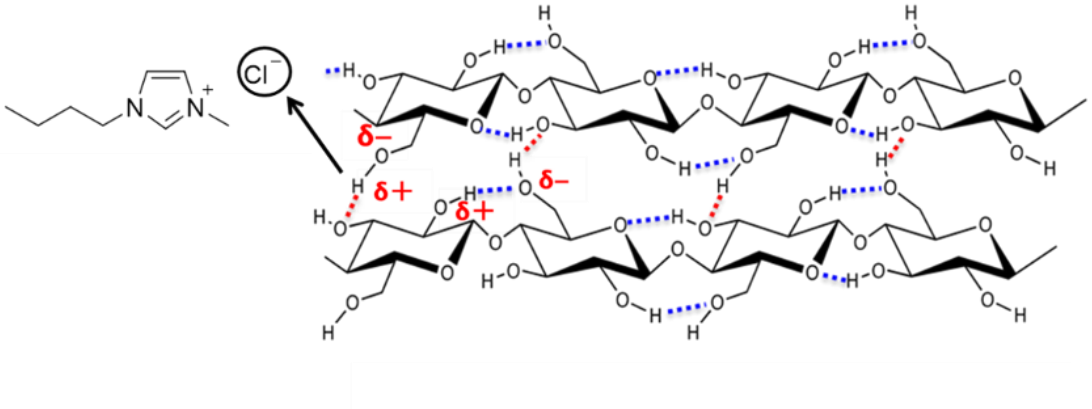



Fig. 1-10 Mechanism of cellulose dissolution by ionic liquid

**Properties of ionic liquids as cryoprotectants**

Ionic liquids have various properties. Here, we describe the interaction with water and toxicity to cells, which are particularly important as cryopreservation agents.

**· Interaction with water**

polarity

In order to know the polarity of an ionic liquid, a method of estimating the degree of interaction from the shift of the maximum absorption wavelength of the dye is used. ET(30) values using Reichadt's dyes are widely used. The Kamlet-Taft parameter is used as the most accurate method for evaluating the polarity of ionic liquids. The Kamlet-Taft parameters can be calculated by dissolving three dyes (Reichadt's dyes, NA, and DENA) in an ionic liquid and substituting the maximum absorption wavelength in the visible region into the following equation. The obtained polar parameters are hydrogen bond donating (α value), hydrogen bond accepting (β value), and ambipolar parameter (π* value)[33].

$$\pi * = 8.641 - 0.314\nu DENA$$

$$\beta = \frac{[1.035\nu NA - \nu DENA + 2.64]}{2.80}$$

$$\alpha = 0.0649ET(30) - 0.72\pi * -2.03$$

$$ET(30) = 0.9986(2.86\nu Reichardt's\ dye) - 8.6878$$

The α, β, and π* values of the ionic liquid and water used this time are shown below.

| | α value | β value | π* value |
|---|---|---|---|
| [$OE_2$eim]OAC | 0.50 | 1.13 | 0.93 |
| $H_2O$ | 1.17 | 0.14 | 1.09 |

Hydrogen bond capacity

Water ($H_2O$) is a typical polar molecule and easily forms hydrogen bonds with other polar molecules and ions. Hydrogen (H) bound to a highly electronegative atom such as oxygen (O) has a δ+ character and forms H-mediated bonds such as O−H…O with other lone pairs of O and halogens. This is called hydrogen bonding. Hydrogen bonding is a type of dipole-dipole interaction. O–H that donates protons are classified as proton donors of hydrogen bonds, and O and halogens that receive protons are classified as proton acceptors.

Hydrogen bonds are stronger as the hydrogen bond donor (α value) and hydrogen bond acceptability (β value) are higher. Molecules with high hydrogen bond donor (α value) and hydrogen bond acceptability (β value) have stronger hydrogen bonds. However, hydrogen bonds do not become strong between molecules with high hydrogen bond donating properties (α value) or between molecules with high hydrogen bond accepting properties (β value).

DMSO, which is generally used as a cryopreservation agent, interacts with water molecules through partial charges, whereas ionic liquids interact with water molecules through charges. The β value of the Kamlet-Taft parameter is 1.13 for [$OE_2$eim]OAC, which is a type of ionic liquid, and 0.76 for DMSO. Therefore, [$OE_2$eim]OAC is expected to interact with water more strongly than DMSO

and suppress ice crystal formation.

**・Toxicity**

The toxicity of ionic liquids varies depending on their structure. Some are highly toxic and can be used as disinfectants, while others are less toxic than organic solvents such as ethanol and DMSO, but many of them are relatively toxic. The reason for this is thought to be the alkyl chain of the cation. The cell membrane is composed of a phospholipid bilayer and is divided into a hydrophilic part and a hydrophobic part. The hydrophobic part of the alkyl chain of the ionic liquid enters between the phospholipids through hydrophobic interaction with the hydrophobic part of the cell membrane. As a result, ionic liquids show high toxicity because they cut molecular interactions between lipids. In a molecular dynamics simulation (MD simulation), it is known that electrostatic interactions attract cations in ionic liquids to cell membranes, and that alkyl chains of cations interact with phospholipids in cell membranes to accumulate in and destroy cell membranes[50]. Fig. 1-11 shows the penetration of cations into the cell membrane by MD simulation. The cations of the ionic liquids used were 1-octyl-3-methylimidazolium (OMIM) and 1-tetradecyl-3-methylimidazolium (TDMIM). These results confirm that the cation tail permeates the cell membrane in a time-dependent manner. In a series of molecular mechanisms, electrostatic interactions attract cations to the anionic portion of the phospholipid bilayer of the cell membrane. The cations then come into close proximity with the phospholipid bilayer of the cell membrane. After the long chain side of the cation is parallel to the cell membrane, the long chain alkyl penetrates the cell membrane. Accumulation of this process leads to disruption of the cell membrane (Fig. 1-12). In other words, it can be said that the longer the alkyl chain of the cation, the easier it is to accumulate in the cell membrane and the more likely it is to exhibit toxicity. Toxicity was actually investigated using microorganisms and animals, and it was found that in all cases, the longer the alkyl chain, the higher the toxicity[51].

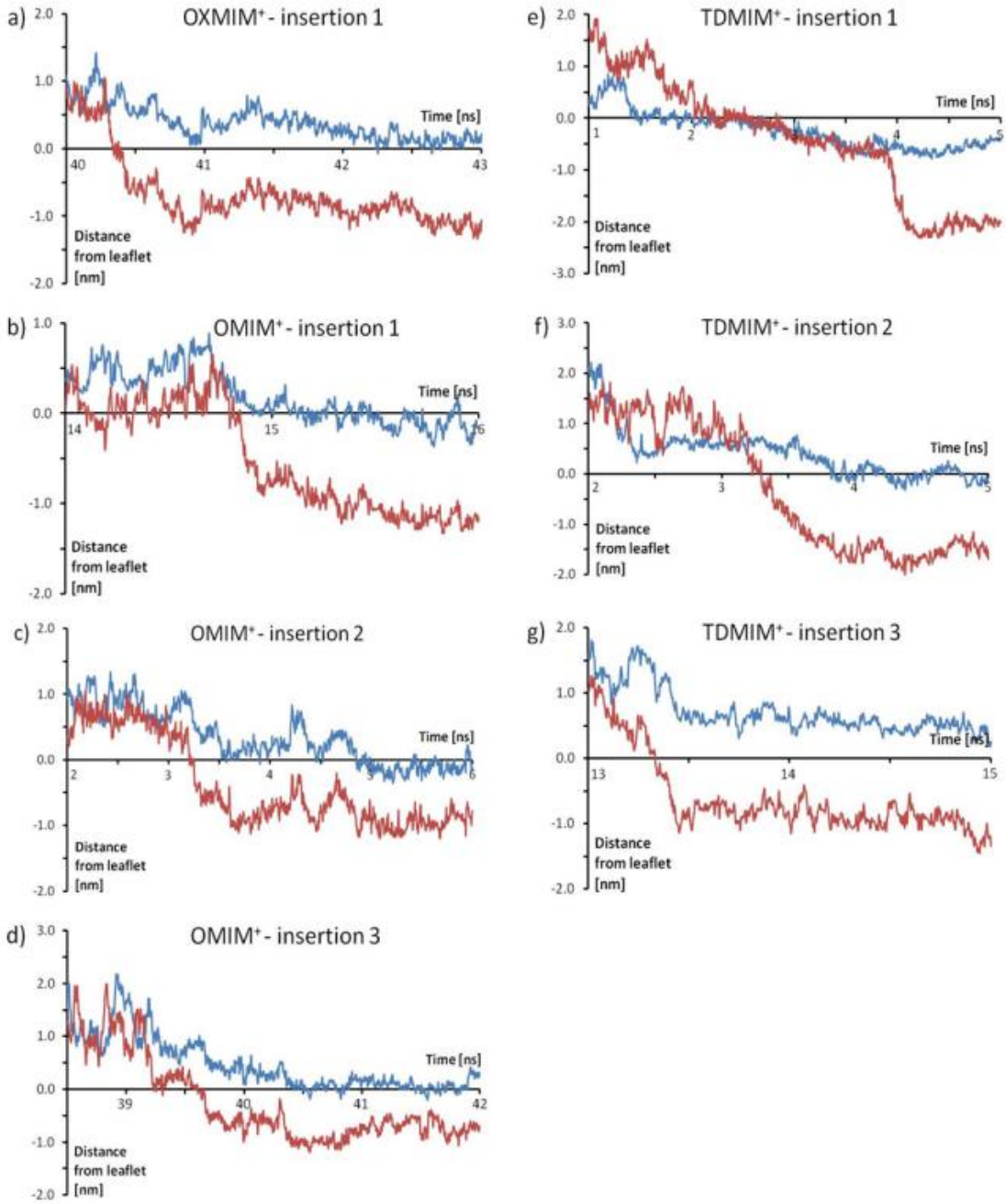



Fig. 1-11 Comparison of the center-of-mass distances of cation head (blue) and tail (red) groups from the membrane layer in z-direction during cation insertion events, respectively. Seven representative instances of cation insertion were selected and are shown in (a) to (g). A tail-first cation insertion mechanism with short cation surface adsorption phases is indicated by these plots.

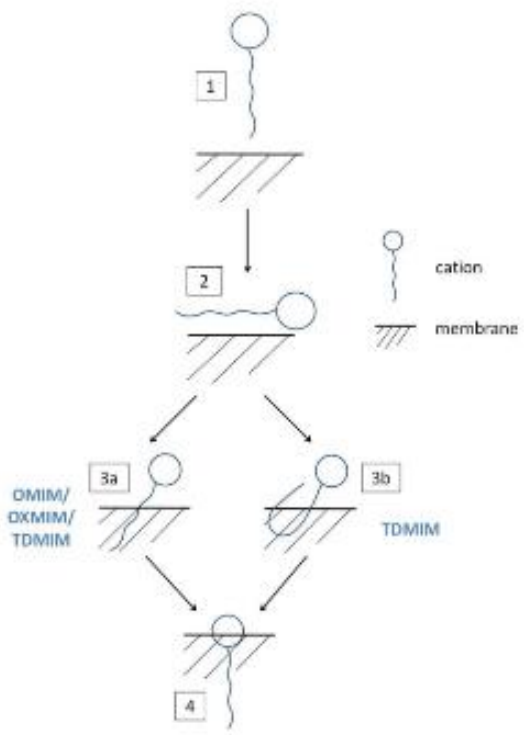



Fig. 1-12 Schematic illustrating the observed cation insertion mechanism.

To cells

Much research has been done on the toxicity of ionic liquids to cells. In particular, toxicity evaluations for human cells are being actively carried out. For example, according to Stepnowski's report[52], for example, IM14 with tetrafluoroborate as an anion species inhibits the growth of Hela cells when added at 0.63 mM. Hexafluorophosphate exhibited no toxicity to cells unless it was added at a relatively high concentration of 10 mM or more. In addition, $[PF_6]^-$ was the most toxic anion when cytotoxicity was evaluated by different anion species. This is thought to be due to the hydrolysis of fluoride having an effect on the cells.

Other evaluations of cytotoxicity using HeLa cells have also been performed. A report by Wang *et al.*[53] shows that salts of tetradecyltrihexylphosphonium bis(trifluoromethylsulfonyl)imide are highly toxic to HeLa cells. In addition, changes in EC50 values with increasing cationic chain length have also been investigated. EC50 refers to the 50% effective concentration. It is the value at which a drug or antibody shows 50% of the maximum response value from the lowest value. The lower the concentration, the higher the effect of the drug. In the case of ionic liquids, the lower this value, the higher the toxicity of the ionic liquid. For example, the EC50 value of 1-ethyl-3-methylimidazolium bromide was 8.4 mM, whereas it was 2.8 and 0.3 mM when the ethyl group was changed to a butyl group or an octyl group, respectively. This indicates that increasing the alkyl chain length of the cation also enhances cytotoxicity in cells.

To animals

Also in animals, the influence of the alkyl chain length of the cation of the ionic liquid is large. For example, Physa acuta, a freshwater pulmonary snail, is known to exhibit toxicity depending on the cation alkyl chain length of the ionic liqui[50]. Interestingly, this is independent of the cationic species such as pyridinium and imidazolium. 1-Alkyl-3-methylimidazolium bromide is shown to have an LC50(lethal) value of 229 mg/L when the alkyl chain length is 4(4 carbon atoms), compared to 56, 8 mg/L when the alkyl chain length is 6 and 8, respectively.

Toxicity evaluation experiments on mice are also being conducted. For example, in an oral administration experiment using [$C_4$mim]Cl, mice administered 175 mg/kg ionic liquid were followed up for 2 weeks and showed no abnormalities in their health. While, they only gained weight. At higher concentrations, 1 of 4 animals died when the concentration was changed to 550 mg/kg. It was also confirmed that 2000 mg/kg [$C_4$mim]Cl died within 1 day to the remaining 3 animals. In addition to oral administration, the toxicity of skin application has also been evaluated[54]. As an experimental method, [$C_4$mim]Cl (2,000 mg/kg) dissolved in distilled water or N,N-dimethylformamide (DMF) was applied to the back of mice to evaluate skin toxicity. When [$C_4$mim]Cl was dissolved in water, erythema and edema were observed for 1–3 days. On the other hand, after 14 days of follow-up, all the mice were alive and no abnormality was observed in their health condition. All five females died when [$C_4$mim]Cl was dissolved in DMF. It also shows that 2 of the 5 males died and the health of the remaining 3 deteriorated.

Thus, ionic liquids are highly toxic and difficult to use for cells and living organisms. Therefore, development of an ionic liquid that is excellent as a new cryopreservation agent but that can solve the safety problem is desired.

## 1.5 Zwitterions

A zwitterion is a covalent bond between a cation and an anion in an ionic liquid. In other words, it is characterized by being a zwitterion, with positive and negative charges in the same molecule. Zwitterions have much stronger covalent bonds than electrostatic interactions in ionic liquids, which raises the melting point. Zwitterions are bulkier than ionic liquids, and because they have positive and negative charges in their molecules, the molecules line up regularly. For this reason, it has drawbacks such as low molecular fluidity and considerably high liquid viscosity. Although zwitterion has the drawback of raising the melting point, it does not migrate under an electric gradient and can maintain ion pairs even when many types of ions coexist. From these characteristics, zwitterion is expected to solve the problems of ionic liquids. Similar to the section on ionic liquids above, thermal properties and ionicity are discussed below, respectively.

**・Thermal properties**

Like ionic liquids, zwitterions strongly depend on their constituent ions. In order to clarify the thermal properties of zwitterions, zwitterions have been synthesized by combining various cations and anions. However, it is said that the factors that lower the melting point are not much different from those of simple ionic liquids.

**・Electrical properties**

Similar to the ionic properties of ionic liquids, it is possible to create user-friendly zwitterions by designing weakly interacting molecules. Both cations and anions use weak ions. For example, if we focus only on the flexibility of the spacer and use an oligooxyethylene chain to lower the melting point, the transference number of ions will drop due to interaction with cations. Therefore, consideration similar to that for the ionic liquid is required.

**Application example of zwitterions**

Zwitterion is being investigated for applications in fields where ionic liquids could not be used. Zwitterion is expected to have infinite possibilities not only in structural properties but also in applications. Here, we will introduce some of them.

**・Electrolytes**

Initially, attention was focused on the use of ionic liquids, which have characteristics such as low volatility and high ionic conductivity, as electrolytes. However, there is a major drawback that the ionic liquid itself moves in response to the potential gradient. In order to overcome this problem, an approach was taken in which the anions and cations of the ionic liquid are linked by covalent bonds to suppress the movement of the ions themselves. This led to the synthesis of zwitterion. Subsequent research led to the successful development of a zwitterion/lithium salt complex exhibiting an extremely high lithium ion transport rate.

**・Biomass solvents for one-pot ethanol fermentation**

At present, bioethanol production from biomass using ionic liquids is attracting attention. In this method, biomass is pretreated with an ionic liquid, washed with the ionic liquid, saccharified with an enzyme, and the resulting glucose is fermented. The washing process is required because ionic liquids are toxic to standard saccharifying enzymes and microorganisms. However, this washing work must be done until the ionic liquid is gone, which poses a problem. These include the use of a large amount of water, the high cost due to the difficulty of recycling the ionic liquid, and the loss of a large amount of biomass during the washing process. In recent years, biomass pretreatment using ionic liquids that

are less toxic to biocatalysts and E. coli resistant to ionic liquids have been developed. With these technologies, this problem can be improved by omitting the washing operation, and all reactions can be performed in one vessel, leading to improved ethanol production (Fig. 1-14). Zwitterion could improve the problem of microbial toxicity, which was a drawback of ionic liquids. Considering the mechanism of toxicity of ionic liquids to microorganisms[50], we focused on carboxylic acid zwitterions among synthesized zwitterions for use in biomass treatment. Carboxylic acid-based zwitterions are similar in structure to carboxylic acid-based ionic liquids that have cellulose-dissolving ability, so we thought that cellulose-dissolving ability could be imparted[48]. In order to realize this one-pot ethanol fermentation, zwitterion, which is liquid at room temperature, was synthesized[55].

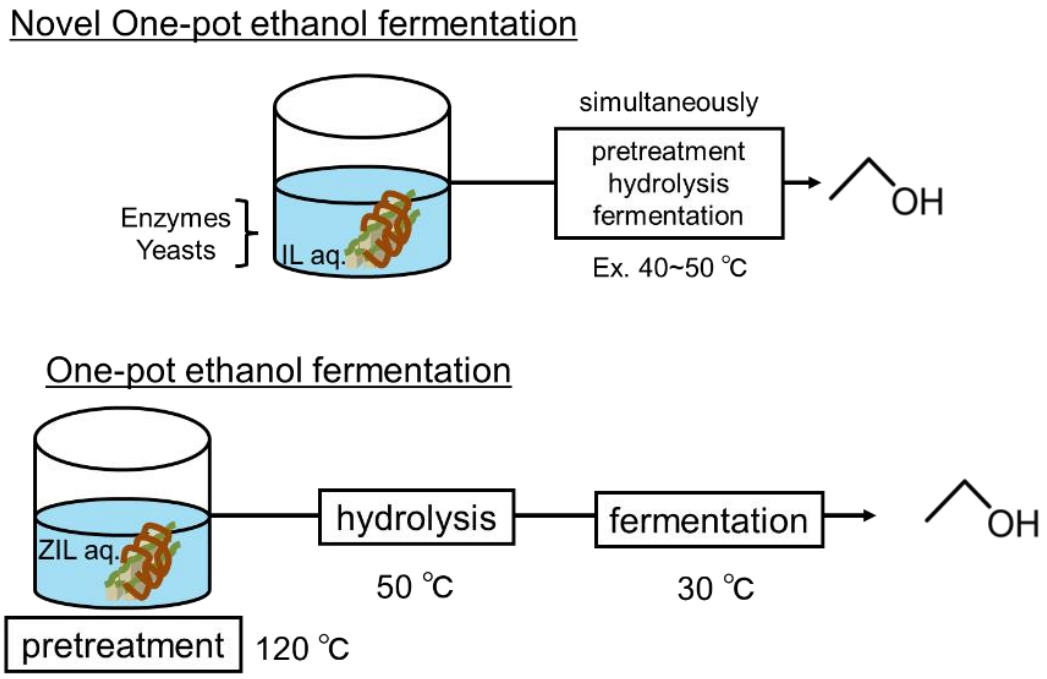



Fig. 1-14 One-pot ethanol fermentation method.

**・Biological application**

DMSO is used as a solvent for various hydrophobic compounds by most researchers and engineers in the life sciences and is recognized as an irreplaceable solvent[19, 22, 56-58]. However, DMSO is somewhat toxic and affects cell behavior in different ways. Qualitatively, DMSO is cell permeable, known to bind to a variety of proteins in the cytoplasm or nucleus[16], directly interrupting their function[18]. These intracellular modifications can indirectly (epigenetically) alter cell behavior[22, 23] and cause apoptosis[24]. Therefore, although DMSO is essential in life sciences, it is not a universal solvent. Zwitterions are biocompatible and dissolve cellulose and related materials that have very low solubility in water and DMSO, showing strong potential as non-aqueous solvents such as solvents for hydrophobic drugs.

In fact, zwitterion dissolved hydrophobic zoledronic acid monohydrate, which is insoluble in neither water nor DMSO[59]. ZIL is a polar solvent, like water and DMSO, and dissolves polar drugs. The difference in dissolution capacity of water and ZIL may be based on the acidity and basicity of hydrogen bonding[55, 60]. Zwitterion dissolved plating agent not soluble in water[59]. Because the anticancer effects of platinum agents (particularly cisplatin) are almost completely abolished by the solvolysis of DMSO[58, 61], these agents could not be stored at high concentrations and were prepared as low-scale working solutions as needed. It was found that zwitterion can be used as a solvent for stock solutions of cisplatin without affecting its anticancer activity. Zwitterion is proposed as the first viable solvent for the stock solution of the plating agent, since solvents other than DMSO do not perform well[58].

**Properties of zwitterions as a cryoprotectants**

Zwitterion have various properties. Here, we describe the interaction with water and toxicity to cells, which are particularly important as cryopreservation agents.

**・Interaction with water**

polarity

In order to know the polarity of a zwitterion, a method of estimating the degree of interaction from the shift of the maximum absorption wavelength of the dye is used. ET(30) values using Reichadt's dyes are widely used. The Kamlet-Taft parameter is used as the most accurate method for evaluating the polarity of zwitterions. The Kamlet-Taft parameters can be calculated by dissolving three dyes (Reichadt's dyes, NA, and DENA) in a zwitterion and substituting the maximum absorption wavelength in the visible region into the following equation. The obtained polar parameters are hydrogen bond donating (α value), hydrogen bond accepting (β value), and ambipolar parameter (π* value)[33].

$$\pi * = 8.641 - 0.314\nu DENA$$

$$\beta = \frac{[1.035\nu NA - \nu DENA + 2.64]}{2.80}$$

$$\alpha = 0.0649ET(30) - 0.72\pi * -2.03$$

$$ET(30) = 0.9986(2.86\nu Reichardt's\ dye) - 8.6878$$

The α, β, and π* values of the zwitterions used this time are shown below.

| | α value | β value | π* value |
|---|---|---|---|
| $OE_2imC_3C$ | 0.46 | 1.12 | 1.10 |
| $OE_2imC_5C$ | 0.38 | 1.14 | 1.02 |
| $H_2O$ | 1.17 | 0.14 | 1.09 |

Other zwitteiron α, β, and π* values are not described in the literature. Since Kamlet-Taft parameters

are measured by adding pigments, individual zwitterions cannot be measured. However, there is a method of pseudo-measuring.

Hydrogen bond capacity

Hydrogen bonds are stronger as the hydrogen bond donor (α value) and hydrogen bond acceptability (β value) are higher. Molecules with high hydrogen bond donor (α value) and hydrogen bond acceptability (β value) have stronger hydrogen bonds. However, hydrogen bonds do not become strong between molecules with high hydrogen bond donating properties (α value) or between molecules with high hydrogen bond accepting properties (β value).

DMSO, which is generally used as a cryopreservation agent, interacts with water molecules through partial charges, whereas ionic liquids interact with water molecules through charges. The β value of the Kamlet-Taft parameter is 1.12 and 1.14 for $OE_2imC_3C$ and $OE_2imC_5C$, which are type of zwitterions, and 0.76 for DMSO. Therefore, zwitterions are expected to interact with water more strongly than DMSO and suppress ice crystal formation.

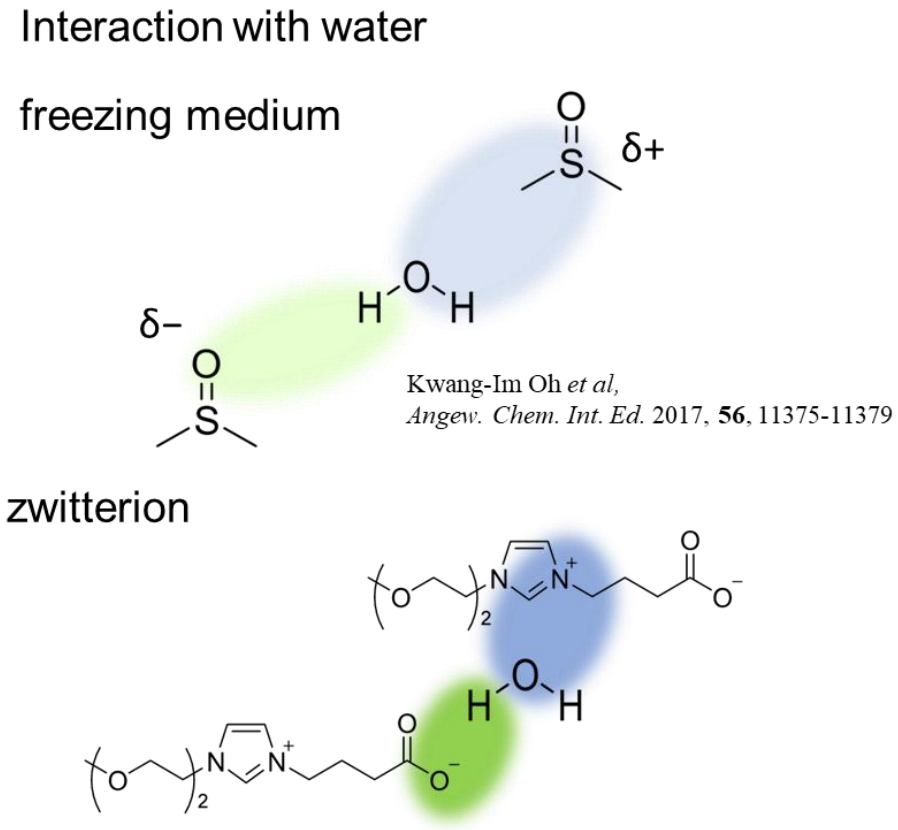



Fig. 1-15 Interaction with water in DMSO and zwitterion.

- **Toxicity**

Zwitterion has a structure in which an anion and a cation are connected, so that the alkyl chain

end of the cation is modified with a carboxylate. Therefore, by introducing a highly polar anion into the alkyl chain of zwitterion, the alkyl chain becomes hydrophilic and does not interact with the hydrophobicity of the cell membrane. Therefore, zwitterion inhibits interactions with cell membranes, and alkyl chains do not intercalate between lipids, resulting in low toxicity to cells[50, 55] (Fig. 1-16).

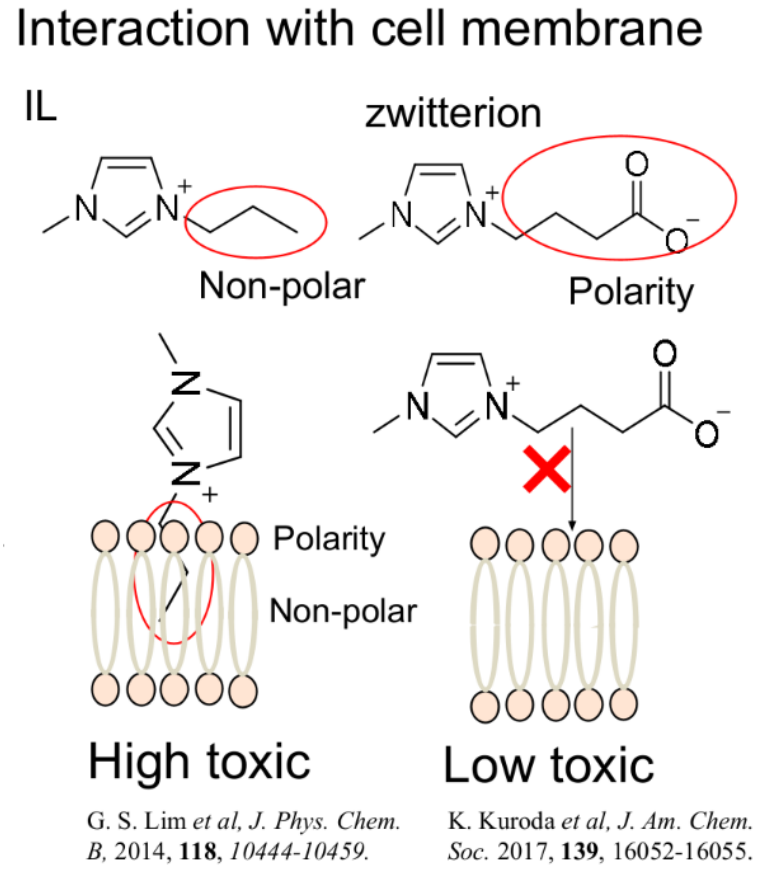



Fig. 1-16 Interaction between ionic liquids and zwitterion cell membranes.

To bacteria

Median effective concentration (EC50) of zwitterion to Escherichia coli is higher than DMSO and ionic liquids[55]. When using 10% imidazolium/carboxylate zwitterion, the viability is higher than using DMSO after 24 hours of cultivation.

To cell

Zebrafish embryos did not die when cultured in 5% zwitterion. On the other hand, when the same concentration of DMSO was used, most of the embryos died[59].

1.6 Objective

Currently, various cells from all over the world are cryopreserved in cell banks and used in

various fields such as pharmaceuticals and regenerative medicine[4]. If cryopreservation technology were not available, all cells have to be cultured without rest, but even then mutations could occur. Therefore, cryopreservation technology that can preserve normal conditions for long periods of time is essential[5]. When cells are frozen directly, ice crystals form inside and outside the cells, physically damaging the cells and causing them to die[9]. In order to cryopreserve cells in a normal state, a freezing medium that suppresses ice crystal formation is required. When cells are frozen by adding cryoprotectants, the extracellular water is first frozen, and then the solutes excluded from the ice crystals undergo a process called cryoconcentration. The concentration of solutes around cells dehydrates them and prevents the formation of deadly intracellular ice crystals[10].

The composition of a typical freezing medium is 10% DMSO, 20% FBS, 70% medium. Optimized commercial freezing media are widely used, but have low cryoprotecting effects on stem cells and non-immortalized cells. For this reason, a new freezing medium is required.

We focused on zwitterion as a new type of cryoprotectant. Zwitterions are a family of ionic liquids in which an anion and a cation are linked by covalent bonds[55, 62]. Also, unlike amino acids, they are aprotic and always have an electric charge. Generally, ionic liquids that always have a similar charge are highly toxic[50, 51, 63, 64], but zwitterion is known to have low toxicity to cells[55, 59, 65-67]. In addition, zwitterion is thought to be used as a cryoprotectant due to its electric charge[12, 16]. Zwitterion can interact more strongly with water than DMSO[55] and can inhibit ice crystal formation[59, 66]. However, zwitterion is a new compound, and it is unclear how it is involved in cryoprotecting effects. In this study, we considered that zwitterion could be an effective cryoprotectant, and investigated the possibilities and limitations of zwitterion as a cryoprotectant. The purpose of this study was to elucidate the cryoprotecting mechanism of zwitterion and to optimize freezing medium containing zwitterion.

In chapter 2, we investigated whether zwitterions actually have a cryoprotecting effect. We also investigated the effect of suppressing ice crystal formation, intracellular dehydration, and toxicity to cells, which is considered to be important as a freezing medium. The cryoprotecting effects of DMSO, a commonly used cryoprotectant, and $OE_2imC_3C$, a type of zwitterion, were compared. In order to understand the cryoprotecting effect level of zwitterion and whether any cells can be cryopreserved, various cells were cryopreserved using zwitterion of various structures under various conditions. The relationship between relative number of living cells and the structure of zwitterion, vitrification, proportion of unfrozen water and osmotic pressure was also investigated. Based on these results, we considered the cryoprotecting mechanism of zwitterion. Based on the clarified cryoprotecting mechanism, we decided to add additives to zwitterion in order to further improve its cryoprotecting effect. A cell-permeable cryoprotectant and a non-cell-permeable cryoprotectant were used as additives. According to the results, we optimized the freezing medium containing zwitterion. In the same way, the effect of suppressing ice crystal formation, intracellular dehydration, and toxicity to

cells were investigated. Finally, we cryopreserved freezing-vulnerable cells in order to compare their performance with commercial freezing medium. The performance of the freezing medium was also investigated by measuring the cell proliferation rate after cryopreservation.

In chapter 3, we considered that polymerizing zwitterion may provide a different cryoprotecting mechanism than that of zwitterion. In chapter 2, it was revealed that dehydration due to osmotic pressure is important in freezing media using zwitterion. However, rapid dehydration caused by hypertonic zwitterion solutions is known to damage cells. In addition, the cryoprotecting effect of zwitterion did not improve unless DMSO, a cell-permeable cryoprotectant, was added as an additive. However, DMSO is toxic to cells. Therefore, it would be ideal to be able to develop a freezing medium with low toxicity to cells. First, a zwitterion polymer (polyZI) was synthesized, and the physical properties of the synthesized polyZI were investigated. The cryoprotecting effects of zwitterion monomer and polyZI were compared. The effect of suppressing ice crystal formation, intracellular dehydration, and toxicity to cells, which is considered to be important as freezing media were investigated. Since it was suggested that polyZI may interact with the cell membrane and form a matrix around the cell membrane, the state of the cells at low temperatures, the shape of the ice crystals formed, and the behavior of the polyZI in solution were investigated. Based on these results, we discussed the cryoprotecting mechanism of polyZI. From there, we optimized the freezing medium using polyZI by varying molecular weight, anionic species, cationic species, degree of ion substitution ($DS_{ion}$), and concentration. The performance of the freezing medium was also investigated by measuring the cell proliferation rate after cryopreservation. The growth rate after cryopreservation using polyZI was poor. In order to improve this, we optimized the cleaning method. Based on the above results, freezing-vulnerable cells were also cryopreserved using polyZI.

In chapter 4, I summarized the research results to date and discussed future prospects.

In this study, I investigated the possibilities and limitations of zwitterion as a cryoprotectant, elucidated the cryoprotecting mechanism of zwitterion, and aimed to optimize this freezing medium to be suitable for all cells.

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## 2. Cryopreservation of cells using zwitterion

### 2.1 Introduction

The composition of a typical commercial freezing media is 10% DMSO, 20% FBS, and 70% medium (D-MEM/FBS/DMSO (70/20/10, v/v/v)). However, while DMSO inhibits ice crystal formation[1, 2], it is toxic to cells[3-5]. Additionally, since FBS contains many unknown substances, it is unclear which substances affected the results, making it impossible to obtain accurate experimental data. Currently, proteins that can replace serum are produced from microorganisms, but there is a risk that viruses may be contaminated during synthesis. Therefore, there is a need for alternatives to DMSO, FBS, and proteins that have cryoprotective properties, but such compounds have not been available.

In addition, some cells cannot be efficiently cryopreserved even if a commercial such as the optimized above freezing media is used. Therefore, cryoprotectants with higher cryoprotecting effects are required. Since the cryoprotectants have been optimized for decades, developing high cryoprotecting effect cryoprotectants requires the use of new, previously unknown compounds.

I focused on the low molecular weight zwitterions as new cryoprotectants[6, 7]. The most important feature of zwitterion is that its structure and physical properties can be designed freely[8], so it has the potential to be used as cryoprotectants. The charged molecules of zwitterions strongly interact with water molecules[8] and thus, more strongly suppress ice crystal formation[6, 7]. Additionally, ionic liquids with similar properties are generally highly toxic[9-12], but zwitterions have low toxicity[6-8, 13-15]. These reasons suggested that zwitterions are considered to be useful cryoprotectants. Furthermore, since zwitterions are chemically synthesized, there is no risk of virus contamination. Zwitterions have never been developed as cryoprotectants because zwitterions are new compounds. In this study, I verified whether zwitterions, whose structure can be freely changed and are expected to have an effect of inhibiting ice crystal formation and low toxicity, can be used as cryoprotectants with high cryopreservation effect and aimed to optimize zwitterions for high cryoprotecting effect and versatility.

### 2.2 Materials and Methods

**Synthesis of zwitterions**

I describe the synthesis method of $OE_2imC_3C$, which is a carboxylate-based zwitterion often used in this study[8].

The stabilizer (dibutylhydroxytoluene) of tetrahydrofuran (THF) used as solvent is removed using aluminum oxide (basic) (Sigma-Aldrich Co., Llc.). Prepare a funnel filled with alumina and pour THF into it to remove the THF impurities. THF is placed in an eggplant flask, the eggplant flask is ice-cooled in an ice bath, and imidazole, sodium hydride, and 1-bromo-2-(2-methoxyethoxy)ethane are added little by little while stirring. This sodium hydride is a hydrophilic substance and is hydrolyzed by contact with water, causing an explosive reaction to generate hydrogen. At this time, heat of reaction is generated, so be careful. After all the contents were added, the mixture was refluxed

at 80 °C for 10 hours. THF is removed from the resulting solution with an evaporator. Distillation is performed to separate the product $OE_2im$ from the solution. $OE_2im$ is recovered by distillation under the conditions of 80 °C and 1 Pa. Add THF as a solvent to $OE_2im$, cool in an ice bath, and add ethyl-4-bromobutyrate while stirring. The mixed solution was refluxed at 80 °C for 16 hours. Wash three times with ethyl acetate to remove unreacted material. After that, the solvent is removed by an evaporator. Anion exchange is performed for 3 days using an anion exchange resin (Amberlite® IRN78 hydroxide form) using methanol (Kanto Chemical Co., Inc.) and water as solvents. After filtering and removing water and methanol with an evaporator, hydrolysis reaction and neutralization reaction occur to obtain the product $OE_2imC_3C$. Methanol is added to the obtained $OE_2imC_3C$, activated carbon is added thereto, and the mixture is stirred all day to remove hydrophobic impurities. After filtering and removing methanol with an evaporator, it was vacuum-dried at 80 °C for 5 hours to obtain $OE_2imC_3C$ as a syrup-like liquid. NMR analysis is performed to confirm the synthesis and confirm its structure.

$OE_2imC_5C$, $C_1imC_3C$, $OE_1imC_3C$, $C_1imC_5C$, $C_4imC_3C$, $C_4imC_5C$, $AimC_3C$, $VimC_3C$, $PyC_3C$, $PyrrC_3C$, and $N_{1,1,4,}C_3C$ were synthesized using the same method with minor modifications[8]. $C_1imC_2C$ was synthesized as previously reported[16].

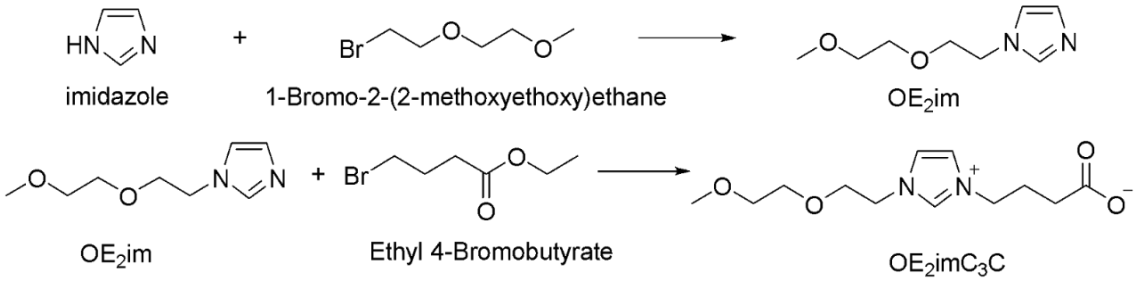


Fig. 2-1 Scheme of synthesis method of $OE_2imC_3C$.

Unlike carboxylate-based zwitterions, sulfonate-based zwitterions proceed in one step and are convenient when alkyl sultones are used. In addition, since no by-products are produced during synthesis, there is no need for purification. Here, I describe the synthesis method of $VimC_3S$ used in this study[17].

The THF used as a solvent is destabilized using aluminum oxide (basic) (Sigma-Aldrich Co., Llc.). Prepare a funnel filled with alumina and pour THF into it to remove the THF impurities. After that, THF was added to the eggplant flask. The vinylimidazole was added little by little while stirring. Then propane sultone was added while cooling in an ice bath. At this time, heat of reaction is generated, so be careful. After adding everything, the mixture was stirred at room temperature for 15 hours. Wash three times with ethyl acetate to remove unreacted material. After that, the solvent is removed by an evaporator. Powdered $VimC_3S$ was obtained by vacuum drying at 80 °C for 5 hours. NMR analysis is performed to confirm the synthesis and confirm its structure.

$C_1imC_3S$, $OE_2imC_3S$, $AimC_3S$, $AimC_4S$ and $VimC_4S$ were synthesized using the same method with minor modifications[17].

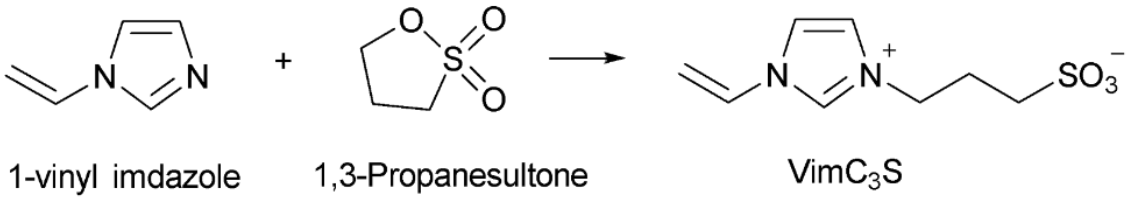


Fig. 2-2 Scheme of synthesis method of $VimC_3S$.

All reagents purchased were used as received unless stated otherwise. The reagents used to synthesize the above-mentioned zwitterions (2-bromoethyl methyl ether, ethyl 6-bromohexanoate, 1-butylimidazole, 1-allylimidazole, 1-vinylimidazole, 2-bromoethyl methyl ether, 1,3-propenesultone, pyridine, pyrroridine, and dimethylbutylamine) were purchased from Tokyo Chemical Industry Co., Ltd., and used as received. Trimethylglycine, L- carnitine, and DMSO were purchased from Tokyo Chemical Industry Co., Ltd., and used as received. Glycerol and sucrose were purchased from Nacalai Tesque, Inc., and used as received.

**Cells**

I received human normal fibroblast (hNF), mouse normal fibroblast (mNF) derived from C57BL/6-EGFP mice, WM266.4 human melanoma cells (WM), MDA-MB-231 human breast cancer cells (MDA), B16F10 mouse melanoma cells, and 4T1 mouse breast cancer cells for kind gifts from professor Erik Sahai (The Francis-Crick Institute, UK). I received BOSC human kidney cells, PC9 human lung cancer cells, Mardin-darby canine kidney cells (MDCK) for kind gifts from professor Michiyuki Matsuda (Kyoto University), professor Seiji Yano (Cancer Research Institute of Kanazawa University), and professor Etsuko Kiyokawa (Kanazawa Medical University), respectively. I received HL-60 human promyelocytic leukemia cells for kindly gifted from professor Atsushi Hirano (Cancer Research Institute of Kanazawa University). I received K562 human chronic myelogenous leukemia cells and Vn1919 neuroglial and neuronal character co-expressing ependymoma cells used in the previous studies[18, 19]. I purchased OVMANA human ovarian tumor cells from the Japanese Collection of Research Bioresources.

**Culture**

hNF, mNF, BOSC, WM, MDA, PC9, MDCK, B16F10, 4T1 and Vn1919 were grown and maintained as monolayer cultures at 37 °C in 5% CO2 humidified atmosphere, using Dulbecco's modified Eagle's medium (high glucose with L-glutamine and phenol red, Fujifilm Wako Pure Chemical Corporation) supplemented with 1 vol% penicillin–streptomycin solution (×100) (Fujifilm

Wako Pure Chemical Corporation) and 10 vol% FBS (Sigma-Aldrich Co., Llc.). OVMANA was grown and maintained as monolayer cultures at 37 °C in 5% CO2 humidified atmosphere, using RPMI, Nacalai Tesque, Inc. supplemented with 1 vol% penicillin–streptomycin solution and 10 vol% FBS. The cells were sub-cultured every 4–6 days with trypsin solution (0.5 w/v% trypsin-5.3 mmol/L EDTA・4Na solution without phenol red (×10), Fujifilm Wako Pure Chemical Corporation).

HL-60 and K562 were grown and maintained as floating cultures at 37 °C in 5% CO2 humidified atmosphere, using Roswell Park Memorial Institute medium (RPMI, Nacalai Tesque, Inc.) supplemented with 1 vol% penicillin–streptomycin solution and 10 vol% FBS.

These cells were sub-cultured every 4–6 days.

**Cryopreservation**

Cells ($1 \times 10^6$ cells) With or without trypsin treatment were harvested and centrifuged (100 × G, 5 min at room temperature). Following removing the supernatant of samples, 100 μL of freezing media were added and pipetted gently. The samples were stored in a box (Mr. Frosty, Thermo Fisher Scientific Inc.) in a –85 °C deep freezer for 3–5 days. For the samples thawing, culture media incubated at 37 °C were added to the frozen samples. The relative number of living cells was calculated by counting using a hemocytometer (Fukaekasei Corporation and Watson Corporation) after staining using trypan blue (Fujifilm Wako Pure Chemical Corporation) and calculated.

The commercial freezing medium employed was Culture Sure freezing medium (DMSO-containing, Fujifilm Wako Pure Chemical Corporation). This is used as one of the representative commercial freezing media and is suitable for comparison in cryoprotecting effect. In this study, the relative number of living cells was adopted because the absolute number of living cells was variable, based on biological variation, even when experiments are repeated under the same conditions and the commercial is used. The absolute numbers of living cells obtained by the commercial were approximately associated with those obtained by the sample solutions. The results still contain a certain amount of error based on the biological variation but are sufficient to suggest rough trends and relations.

$$\text{The relative number of living cells} = \frac{\text{Counted living cell number (sample)}}{\text{Counted living cell number (commercial)}}$$

**Cryopreservation solution adjustment**

Freezing media are typically prepared with concentrations based on volume/volume using pipettes; however, $OE_2imC_3C$ aqueous solution are difficult to prepare at volume/volume due to their high viscosity. As a result, they were prepared at volume/weight in the present study. The zwitterion and DMSO solutions were prepared via mixing with ultrapure water or medium. When preparing the freezing medium, the amount of culture medium, FBS, and water were measured by volume using

pipettes. The number of zwitterions, DMSO, an ionic liquid, glycerol, and sucrose were measured by weight using an electronic balance unless I note.

**Growth rate**

All cells after cryopreservation were seeded in 6-well plates. K562 cells were counted sequentially after the indicated days. OVMANA cells were counted after trypsin treatment when most proliferating cells reached 80% confluence.

**Toxicity of cryoprotectants**

Cells ($1 \times 10^5$ cells) after trypsin treatment were harvested and centrifuged (100 × G, 5 min at room temperature). Following the removing the supernatant of samples, 100 μL of the samples were added and pipetted gently. The cells were then incubated at room temperature (15–25 °C) and 0 °C as floating cells in samples. After 60 min in a floating state, the dead cell rate was calculated by counting using a hemocytometer and staining with trypan blue. Dead cell rate was defined as the following equation.

$$\text{Dead cell ratio (\%)} = \frac{\text{Number of dead cells}}{\text{Number of living cells} + \text{Number of dead cells}}$$

**Cell volumes in cryoprotectants**

Cells ($1 \times 10^5$ cells) after trypsin treatment were harvested and centrifuged (100 × G, 5 min at room temperature). Following the removing the supernatant of samples, 100 μL of samples were added and pipetted gently. The cells were then incubated at room temperature (15–25 °C) or 0 °C as floating cells in samples After 5 min in a floating state, images of the cells were captured with an optical inverted microscope (IX83, Olympus Corporation). The cell radii were estimated using the "ImageJ" software (ImageJ 1.52p, Wayne Rasband, National Institutes of Health, USA). The relative cell volume was calculated using the measured cell radii, relative to the cell volume in phosphate-buffered saline (PBS). The relative cell volume was defined as the following equation.

$$\text{Relative cell volume (\%)} = \frac{\text{Cell volume in sample } (\mu m^3)}{\text{Cell volume in PBS } (\mu m^3)} \times 100$$

**Water content of cells**

Cells ($1 \times 10^7$ cells) after trypsin treatment were harvested and centrifuged (100 × G, 5 min at room temperature). Following removing the supernatant of samples, the weight of the cells was measured. Thereafter, the cells were dried in vacuo (1 Pa) and the weight was measured. The water content of original cells and water content of cells in water/$OE_2imC_3C$ (90/10, v/w) was calculated as following equations.

$$Water\ contect\ of\ \text{oringinal}\ cells(\%) = \frac{\text{Cell weight before drying} - \text{Cell weight after drying}}{\text{Cell weight before drying}} \times 100$$

$$\text{Water content of cells in}\ \frac{\text{water}}{\text{OE2imC3C}}\left(\frac{90}{10}, \frac{\text{w}}{\text{v}}\right)(\%) = \frac{\text{Relative cell volume} - (100 - \text{Water content of original cells})}{\text{Relative cell volume}}$$

**Physical state of cryoprotectants under cryogenic temperature**

The phase behavior of the sample under cryogenic temperature was investigated using DSC (DSC-60A plus, Shimadzu Corporation). DSC measurements were performed under the following conditions: cooling to −100 °C at a cooling rate of −1 °C/min followed by heating to 25 °C at a heating rate of 5 °C/min.

**The proportion of unfrozen water**

The proportion of unfrozen water in the sample was estimated from the area of the melting peak at ~0 °C using DSC. The proportion of unfrozen water was defined as the following equation.

$$\text{Proportion of unfrozen water in solution}\ (\%) = 100 - \frac{\text{Melting heat of the sample solutions [J/g]}}{\text{Melting heat of water (265 [J/g])} \times \text{water proportion in the solution}} \times 100$$

**Statistical analysis**

The experimental data were subjected to one-way ANOVA followed by Dunnett test. These tests were performed by GraphPad Prism9.

Biological triplicate used cells harvested from the different dishes to reduce experimental errors caused by individual differences in the organisms used as experimental samples. Experimental triplicate used cells harvested from the same dish to reduce errors caused by experimental manipulation.

2.3 Cryopreservation of cell lines with water/$OE_2imC_3C$ (95/5, v/w).

Actually I investigated whether water/$OE_2imC_3C$ (95/5, v/w) can be used as a universal freezing medium. Nine cell lines from various animals and parts were cryopreserved, and the relative number of living cells after thawing was evaluated (Fig. 2-3). The cryoprotecting effect was found to be

significantly different depending on the cell line. For example, hNF had the highest relative number of living cells of 1.10 while the number of living cells after cryopreservation with the commercial cryoprotectant was 1.00. In contrast, BOSC had the lowest relative number of living cells of 0.20. These results indicate that the differences may not be based on the parental mammal species. Therefore, hNF with similar viability compared to commercial products, mNF with slightly lower viability, and BOSC with very low viability were selected as representative examples, and the differences in $OE_2imC_3C$ cryoprotecting effects were investigated.

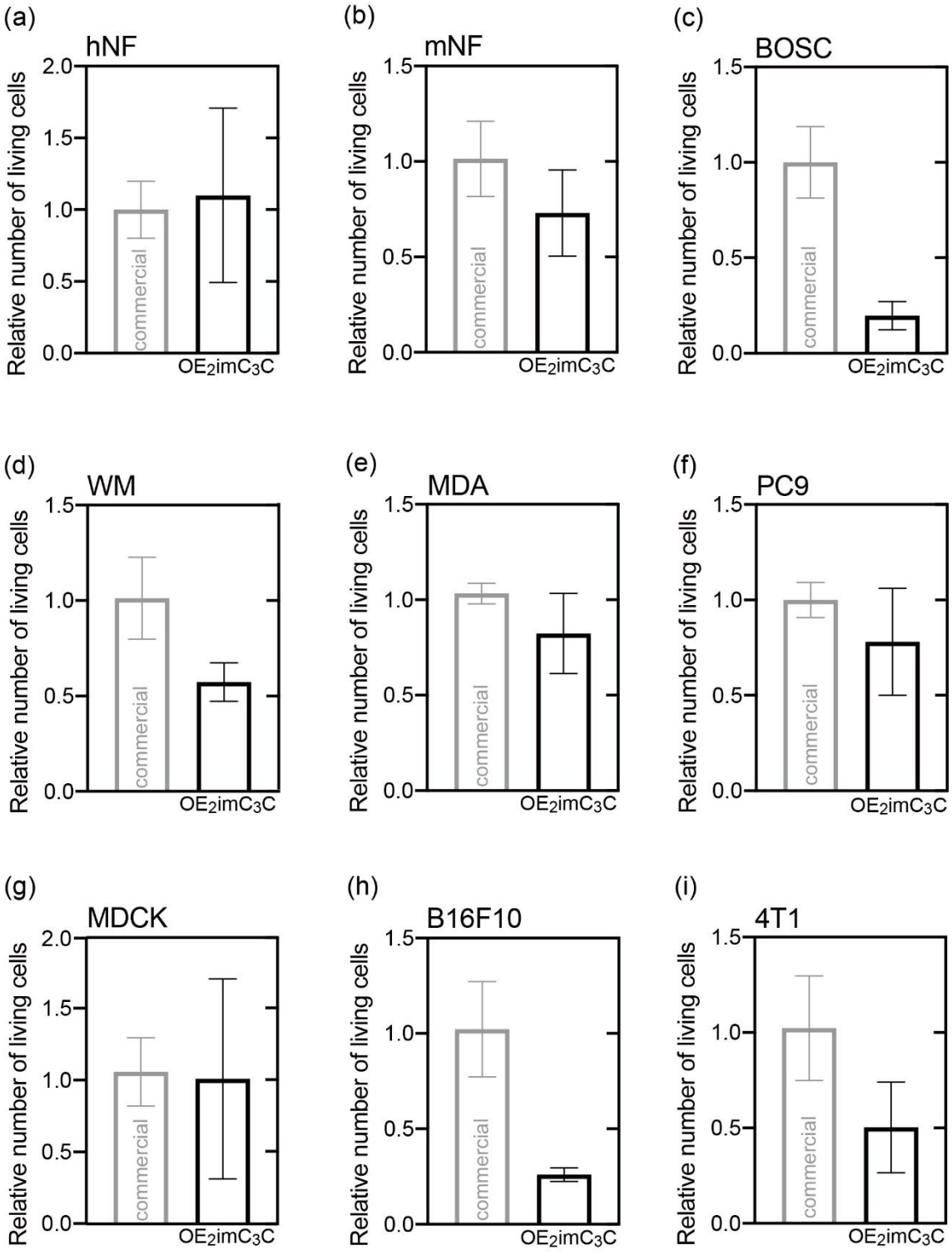


Fig 2-3 The relative number of living cells after cryopreservation indicated cells using a commercial freezing medium and water/$OE_2imC_3C$ (95/5, v/w). (n = 3, biologically triplicate, one-way ANOVA) Commercial is Culture Sure freezing medium (Fujifilm Wako Pure Chemical Corporation). Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants.,

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## 2.4 Establishment of experimental method

The reason why the cell viability when using water/$OE_2imC_3C$ (95/5, v/w) was lower than when using the commercial may be that the conventional cryopreservation method was not suitable. I thought that the cell viability could be further improved by optimizing the cryopreservation method using zwitterion aqueous solutions. Therefore, I considered about cell counting, cryopreservation duration and thawing.

### Cell count

The relative number of living hNF cells when cryopreserved using water/$OE_2imC_3C$ (95/5, w/v) is significantly different from previous study[6]. This is due to the different methods of measuring the number of living cells after cryopreservation. I examined the difference between counting with ®Countess after staining with trypan blue, as used in the previous study, and counting with a hemocytometer after staining with trypan blue, as in this study. As a result, the cell viability when cryopreserved using a commercial was 82% when measured by ®Countess and 105% when measured by a hemocytometer. The relative number of living cells after cryopreservation with 5, 10, 15, 25% (w/v) $OE_2imC_3C$ aqueous solutions were 0.87, 0.96, 0.87, 0.96, respectively when measured by ®Countess and 0.53, 0.64, 0.47, 0.62, respectively (Fig. 2-4). For these reasons, when counting with ®Countess, the values were often higher than when counting with a hemocytometer. Probably, counting with a hemocytometer is correct, and that counting with ®Countess incorrectly counts dead cells as live cells[20]. Therefore, in this study, the relative number of living cells was measured using a hemocytometer.

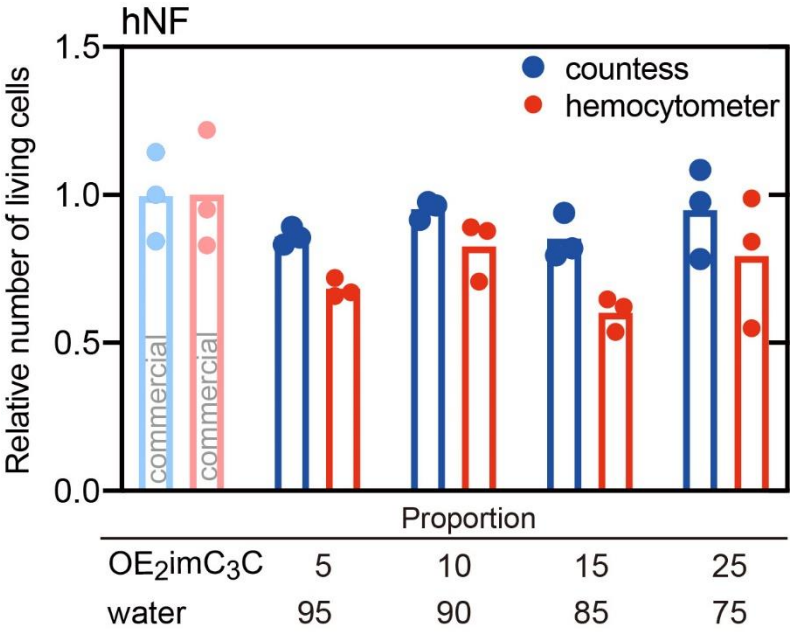



Fig. 2-4 The relative number of living hNF cells after cryopreservation counted by using ®Countess II FL and hemocytometers. The cells were taken from the same samples and subjected to counting by both methods. (n = 3, experimentally triplicate) Commercial is Culture Sure freezing medium (Fujifilm Wako Pure Chemical Corporation). Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

**Immersion time in cryoprotectant**

To investigate the effect of the immersion time in the freezing medium, I compared hNF cells frozen immediately after adding the freezing medium and hNF cells frozen after standing for 60 minutes after adding the freezing medium. As a result, the relative number of living hNF cells was high for all freezing media when frozen immediately after adding the freezing media (Fig. 2-5). However, when frozen after standing for 60 minutes after adding the freezing media, the relative number of living hNF cells of the commercial decreased significantly, but that of $OE_2imC_3C$ aqueous solution decreased slightly. This result may be related to the toxicity of the freezing medium itself. The shorter the time of immersion in the freezing medium, the less the toxicity for cells of the freezing medium. Therefore, I cryopreserve cells immediately after adding the freezing medium in this study.

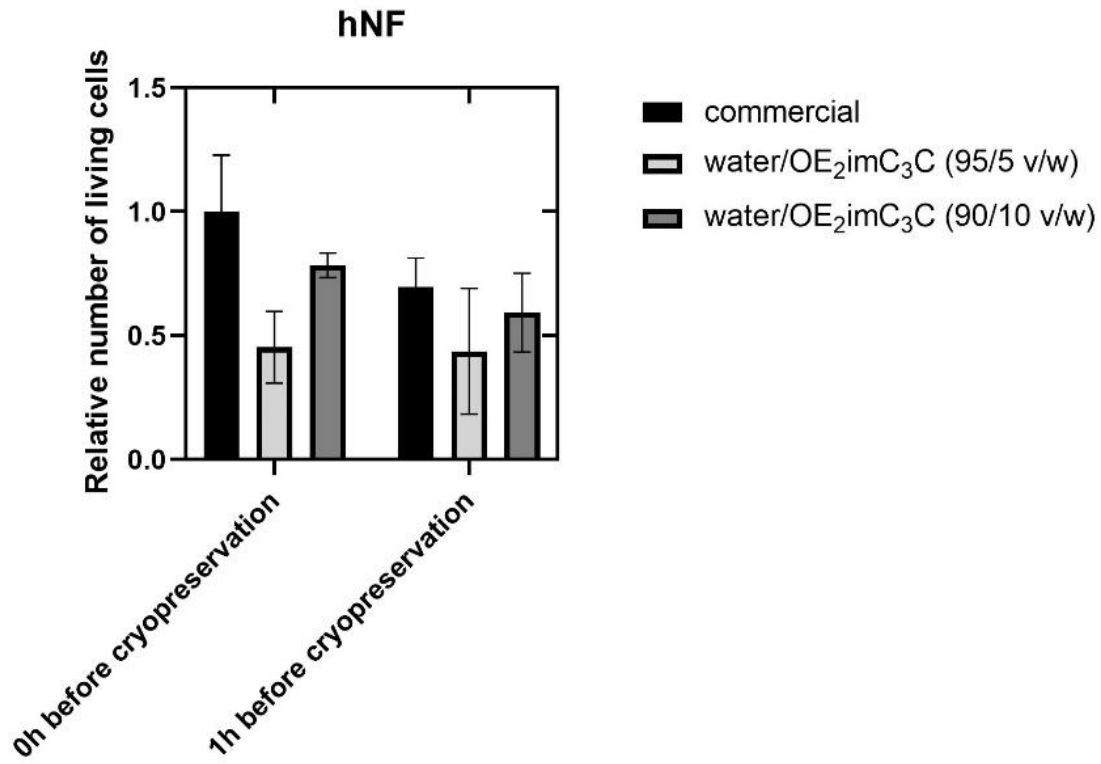



Fig. 2-5 The relative number of living hNF cells after cryopreservation with the indicated freezing media when the time of immersion in the freezing media is changed. (n = 3, experimentally triplicate) There is no statistical significance between the samples ($p > 0.1$) Commercial is Culture Sure freezing medium (Fujifilm Wako Pure Chemical Corporation).

**Thawing**

To investigate the effect of different thawing methods, I compared hNF thawing in medium warmed to 37 °C after cryopreservation and hNF thawing by standing at room temperature. As a result, the relative number of living hNF cells was high for all freezing media when thawing in medium at 37 °C after cryopreservation (Fig. 2-6). However, when thawed by standing at room temperature, the relative number of living hNF cells of $OE_2imC_3C$ aqueous solution was significantly reduced, while that of commercial did not change significantly. This result may be related to the vitrification of the freezing medium. If the freezing medium is vitrified, the faster the thawing time, the less likely it is to recrystallize. Therefore, I thaw cells in medium warmed to 37 °C after cryopreservation in this study.

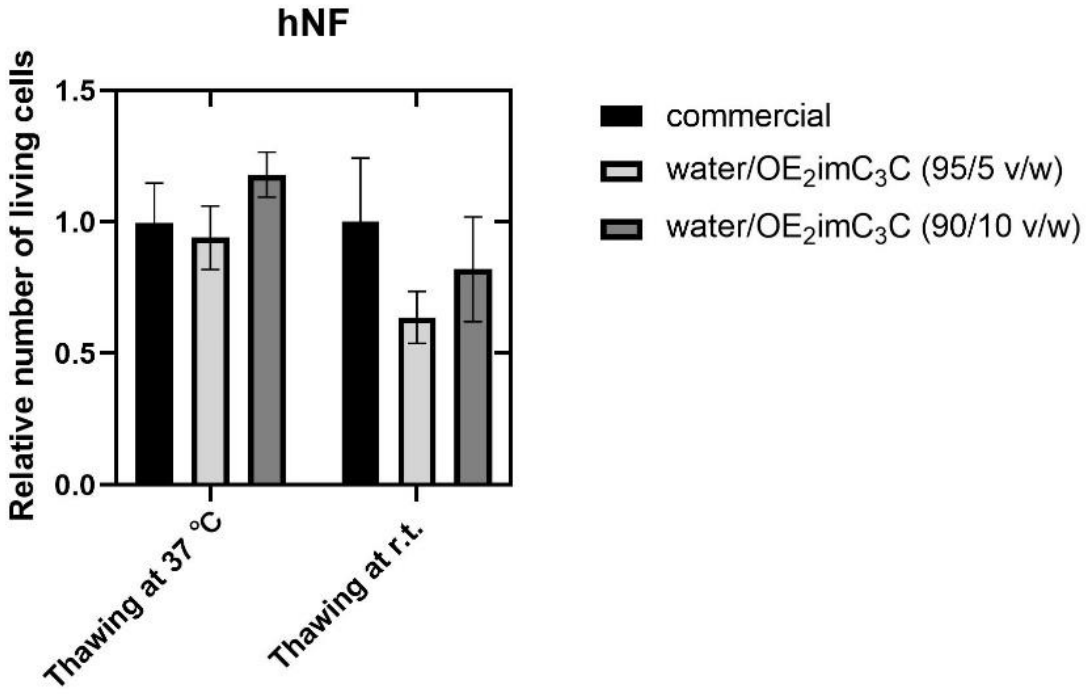



Fig. 2-6 The relative number of living hNF cells after cryopreservation with the indicated freezing media when the thawing time and temperature is changed. (n = 3, experimentally triplicate) There is no statistical significance between the samples ($p > 0.1$) Commercial is Culture Sure freezing medium (Fujifilm Wako Pure Chemical Corporation).

**Cryopreservation duration**

Cryopreservation is usually done for long-term storage. However, for this experiment, I have been conducting the experiment under the condition thawed after cryopreservation it for two days. However, it is desirable to thaw as soon as possible for the experiment to proceed efficiently. Therefore, I optimized the cryopreservation duration. BOSC cells were cryopreserved using a commercial and $OE_2imC_3C$ aqueous solution, and then thawed every 19, 24, 48, and 72 hours. As a result, the relative number of living BOSC cells has not change since then 48 hours (Fig. 2-7). In this study, I decided to thaw after 48 hours.

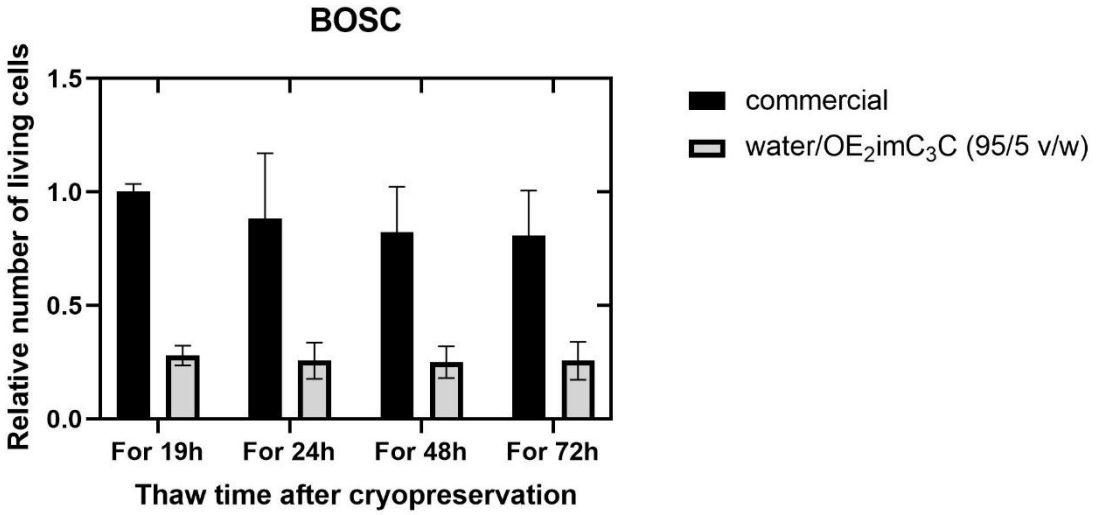



Fig. 2-7 The relative number of living BOSC cells after cryopreservation with the indicated freezing media when the time to cryopreserve is changed. (n = 3, experimentally triplicate) There is no statistical significance between the samples ($p > 0.1$) Commercial is Culture Sure freezing medium (Fujifilm Wako Pure Chemical Corporation).

2.5 Comparison of medium and water as dilute freezing media.

In previous studies, a simple solution containing only $OE_2imC_3C$ and water was used for cryopreservation[6]. This is an oversimplification and may not have improved cryoprotecting effect. I compare it with a similar composition to the commercial. A representative commercial freezing medium composition is D-MEM/FBS/DMSO (70/20/10, v/v/v). Since $OE_2imC_3C$ aqueous solution functions as a freezing medium, $OE_2imC_3C$, instead of DMSO, speculated to inhibits ice crystal formation. Cells were cryopreserved using DMSO changed to $OE_2imC_3C$. A system in which D-MEM was replaced with water was also cryopreserved. Since it is not known how FBS inhibits ice crystal formation, I cryopreserved in a system that did not contain FBS. The relative number of living cells with D-MEM/FBS/DMSO (70/20/10, v/v/v) was 0.99 and that with D-MEM/FBS/$OE_2imC_3C$ (70/20/10, v/v/v) was 0.96 (Fig. 2-8). This suggested that $OE_2imC_3C$ could replace DMSO. On the other hand, in the case of DMSO, the cell viability extremely decreased when the D-MEM or FBS was absent, but in the case of $OE_2imC_3C$, the cell viability did not decrease even without the D-MEM or FBS. These findings suggested that the cryoprotecting mechanism with $OE_2imC_3C$ was different from that with DMSO, and FBS and D-MEM were not related to the cryoprotecting mechanism of zwitterion aqueous solutions. I previously reported that $OE_2imC_3C$ hardly penetrates into cells[6], which is consistent with these results. In the case of DMSO, which is a cell-permeable cryoprotectant, it penetrates into cells and causes them to swell. If it swells too much, it will be destroyed, so the inside of the cell must be dehydrated. Non- cell-permeable components in the medium, such as NaCl, are

required to dehydrate the cells. Since D-MEM and FBS play this role, the cryoprotecting effect decreased when they were removed. On the other hand, $OE_2imC_3C$ is a non- cell-permeable cryoprotectant and has sufficient osmotic pressure (626 mOsm) compared to the medium (328 mOsm). Since $OE_2imC_3C$ dehydrates cells and suppresses extracellular ice crystal formation, FBS and D-MEM for inhibiting extracellular ice crystal formation may not be necessary. As a result, when $OE_2imC_3C$ was used for cryopreservation, FBS and D-MEM did not improve the cryoprotective effect, and $OE_2imC_3C$ aqueous solution was sufficient. Here, $OE_2imC_3C$ aqueous solution is very advantageous in that it does not contain FBS or D-MEM because FBS contains many unknown substances, so it may be unclear which substances affected the results.

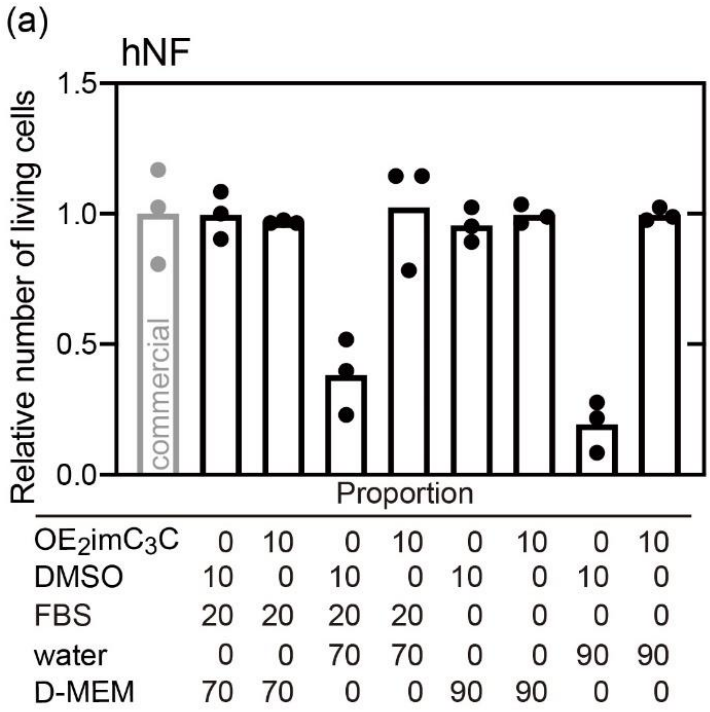



Fig. 2-8 The relative number of living hNF cells after cryopreservation with the indicated freezing media to compare with the cryoprotecting effect of commercial. In this experiment, DMSO was measured in volume. (n = 3, experimentally triplicate) Commercial is Culture Sure freezing medium (Fujifilm Wako Pure Chemical Corporation). Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

Similarly, mNF and BOSC cells were cryopreserved under the same conditions. As a result, even when using $OE_2imC_3C$ aqueous solution, the relative number of living cells was lower than that of commercial, but the same tendency was obtained (Fig. 2-9). From these results, I concluded that a simple freezing medium consisting of water and $OE_2imC_3C$ was sufficient for the following

experiments.

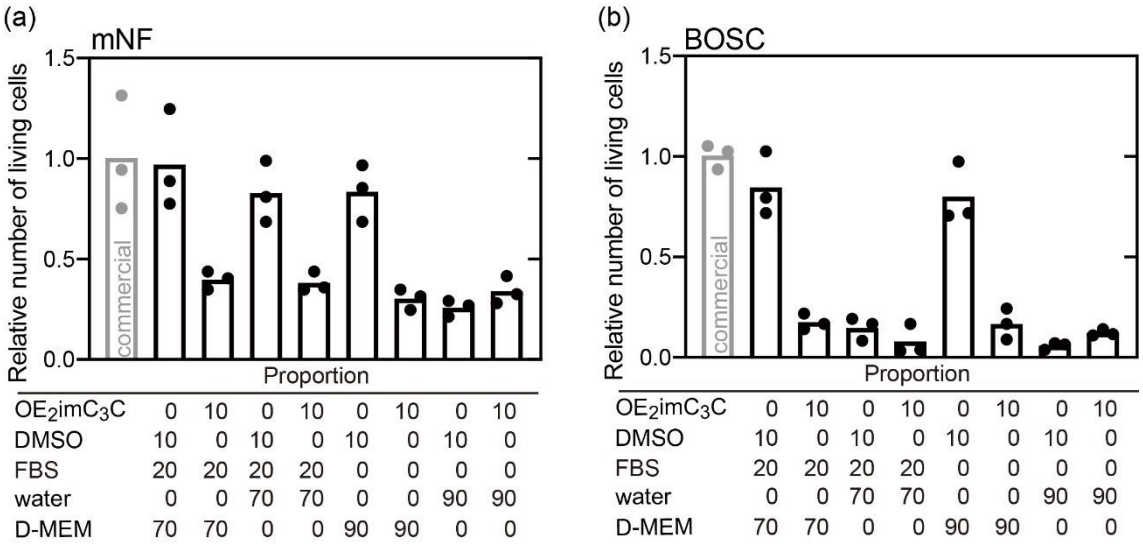



Fig. 2-9 The relative number of living mNF and BOSC cells after cryopreservation with the indicated freezing media to compare with the cryoprotecting effect of commercial. In this experiment, DMSO was measured in volume. (n = 3, experimentally triplicate) Commercial is Culture Sure freezing medium (Fujifilm Wako Pure Chemical Corporation). Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

2.6 Optimal concentration comparison of $OE_2imC_3C$.

**Cell viability**

Since the components of the freezing medium were optimized, I investigated the optimal $OE_2imC_3C$ concentration to improve the cryoprotecting effect. The relative number of living hNF cells when using 5, 10, 15, 25, 35, 50% (w/v) $OE_2imC_3C$ aqueous solutions were 0.47, 0.94, 0.90, 0.62, 0.17, 0.18, respectively (Fig. 2-10). In the case of hNF, the optimal value was around 10-15% (w/v).

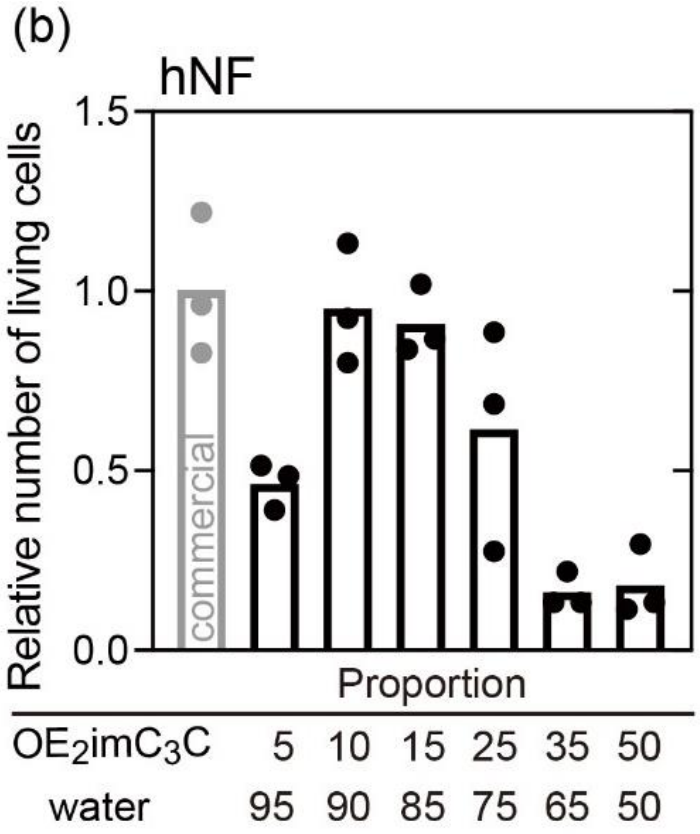



Fig. 2-10 The relative number of living hNF cells after cryopreservation with the indicated freezing media to search for the optimum concentration of $OE_2imC_3C$. (n = 3, experimentally triplicate) Commercial is Culture Sure freezing medium (Fujifilm Wako Pure Chemical Corporation). Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

Similarly, mNF and BOSC cells were cryopreserved using 5, 10, 15, 25, 35 and 50% (w/v) $OE_2imC_3C$ aqueous solutions. As a result, the relative number of living cells with $OE_2imC_3C$ at any concentration were lower than that of the commercial, but as with hNF, the cell viability was higher when 10 and 15% (w/v) $OE_2imC_3C$ aqueous solutions were used. (Fig. 2-11). In mNF and BOSC, the cell viability at 25% (w/v) was similar to that at 10 and 15% (w/v).

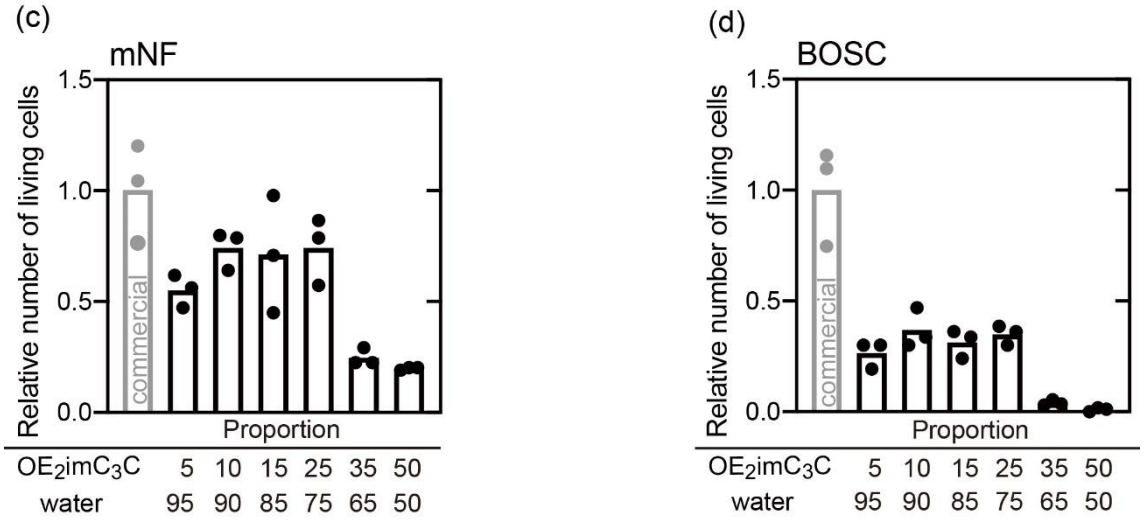



Fig. 2-11 The relative number of living mNF and BOSC cells after cryopreservation with the indicated freezing media to search for the optimum concentration of $OE_2imC_3C$. (n = 3, experimentally triplicate) Commercial is Culture Sure freezing medium (Fujifilm Wako Pure Chemical Corporation). Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

**Cell volume**

Since $OE_2imC_3C$ is a non-cell-permeable cryoprotectant, intracellular dehydration was insufficient when the concentration was 5% (w/v) or less, and ice crystals were formed in the cells, resulting in a decrease in viability. At this time, the cell volume in water/$OE_2imC_3C$ (90/10, w/v) is equivalent to the cell volume in commercial, and they are sufficiently dehydrated (Fig2-12).

The isotonic hosphate-buffered saline (PBS) was used as a control. The osmotic pressures of 5, 10, 15 and 25% (w/v) $OE_2imC_3C$ aqueous solutions were 381, 626, 921 and 1501 mOsm, respectively, and were proportional to the concentration. The cell volume is basically proportional to the osmotic pressure, and in fact, a proportional relationship was observed up to 10% (w/v) between the volume of hNF and the osmotic pressure of the cryoprotectant[21, 22]. However, at osmotic pressures above 10% (w/v), the change in cell volume became smaller (Fig. 2-13). It is thought that the cell volume may be the limit in the case of 10% (w/v).

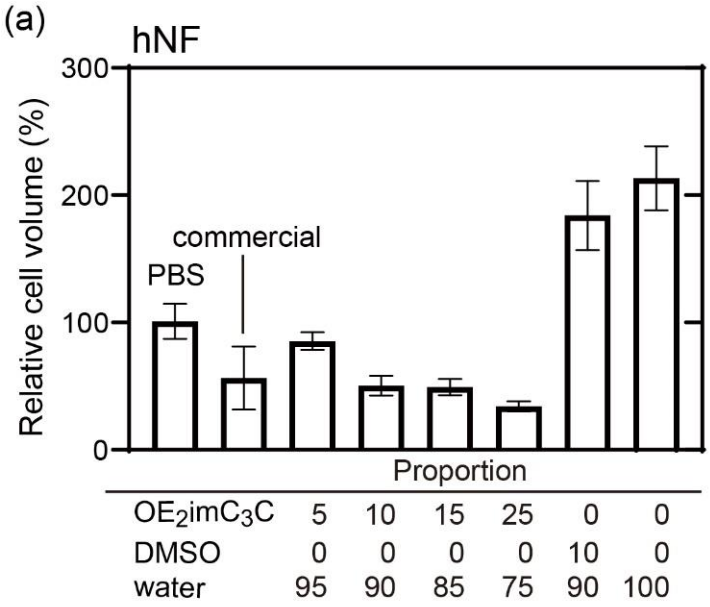



Fig. 2-12 The relative hNF cell volumes in the indicated concentration after 5 min immersion. The hNF was immersed as floating cells in the $OE_2imC_3C$ aqueous solutions after trypsin treatment. (n = 3, biologically triplicate, one-way ANOVA) There is no statistical significance between the samples ($p > 0.1$) Commercial is Culture Sure freezing medium (Fujifilm Wako Pure Chemical Corporation). Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

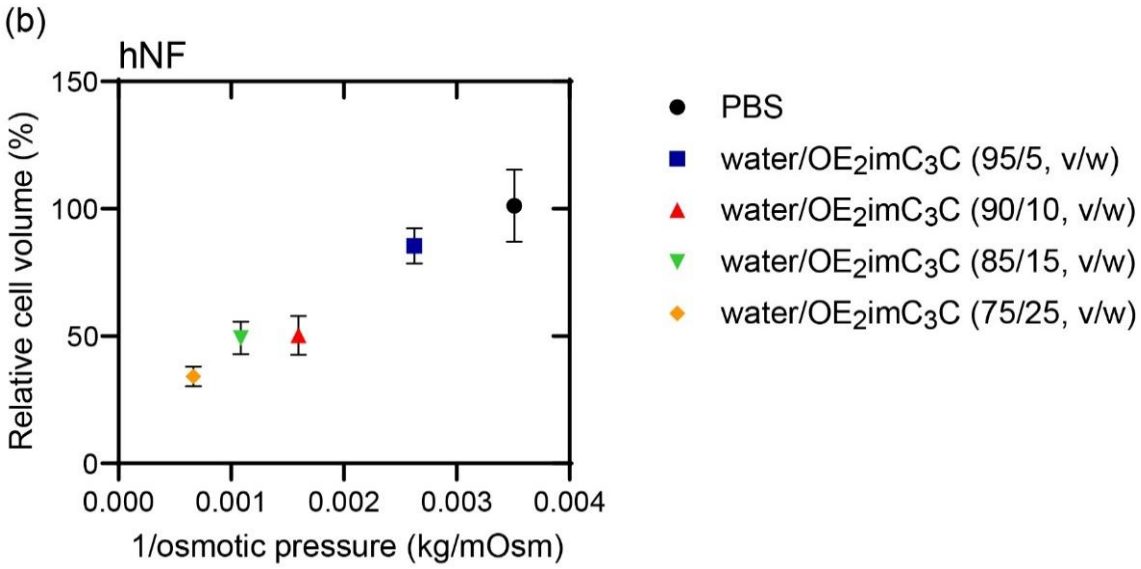



Fig. 2-13 The relation between osmotic pressure and the relative hNF cell volumes in the indicated $OE_2imC_3C$ aqueous solutions and PBS (standardized as 100%) after 5 min immersion as floating cells. The plots correspond to PBS and 5, 10, 15, and 25% (w/v) $OE_2imC_3C$ aqueous solutions (from right to left). (n = 3, biologically triplicate, one-way ANOVA) Material from: Kato, Y. *et al.*, Synthetic

zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

**Dead cell rate**

$OE_2imC_3C$ is said to have low toxicity for cells, but the higher the concentration, the more toxic it becomes. Over 35% (w/v) of $OE_2imC_3C$ was toxic to hNF cells, which may have reduced the living cells (Fig. 2-14). However, even at concentrations as high as 25% (w/v), it was as toxic as commercial. In addition, the shock[23] due to the outflow and inflow of water during freezing and thawing may be one of the reasons why the cell viability decreased from 25% (w/v).

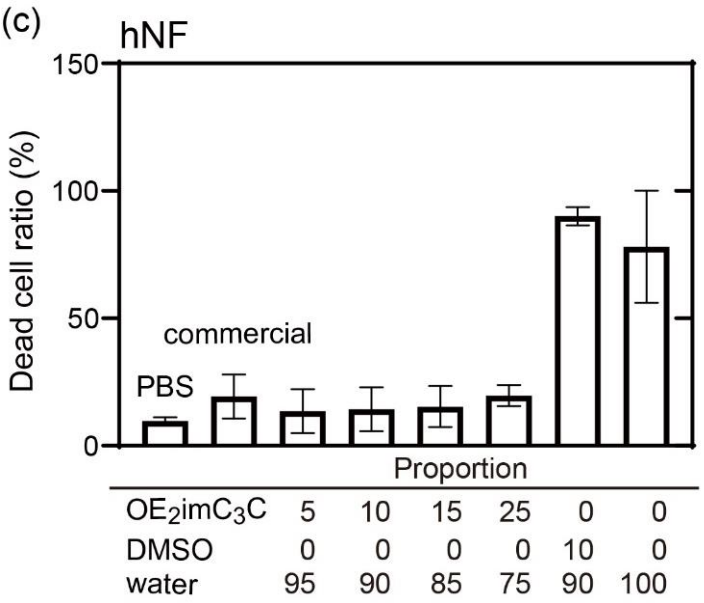



Fig. 2-14 Dead hNF cell ratio in the indicated solutions after 60 min immersion. The hNF cells were immersed as floating cells in the solutions after trypsin treatment. (n = 3, biologically triplicate, one-way ANOVA) There is no statistical significance between the samples ($p > 0.1$) Commercial is Culture Sure freezing medium (Fujifilm Wako Pure Chemical Corporation). Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

Based on the above results, the optimum concentration was determined to be 10%, and the following experiment was conducted.

2.7 Assessment of cell line dependence on cryopreservation with water/$OE_2imC_3C$ (90/10, v/w).

**Cell viability**

Furthermore, three types of cancer cells, WM, MDA, and PC9, were cryopreserved using

water/$OE_2imC_3C$ (90/10, w/v) to confirm whether $OE_2imC_3C$ aqueous solution is versatile as a freezing medium. The cryoprotecting effects differed greatly depending on the cell type, and some cells showed the same viability as when using the commercial and some cells showed a lower viability than when using the commercial. Even at the highest values, the relative number of living cells of mNF, BOSC and WM were only 0.70, 0.37 and 0.24 (Fig. 2-15). Unlike the case of hNF, it was shown that the cryoprotecting effect of the $OE_2imC_3C$ aqueous solution alone is not comparable to that of the commercial even after optimization for the above three types of cells. This reaffirms the need to investigate differences between cell types.

(a)

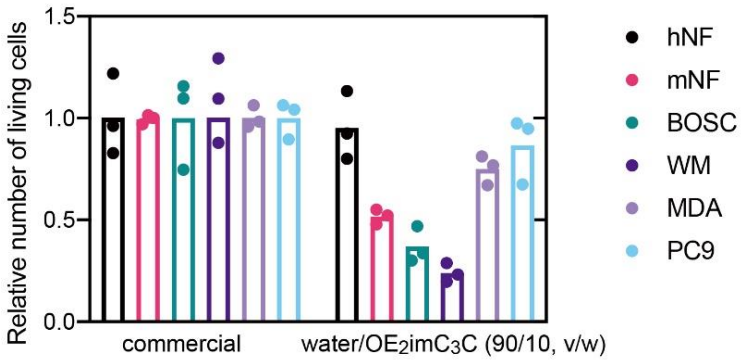



Fig. 2-15 The relative number of living hNF, mNF, BOSC, WM, MDA and PC9 cells after cryopreservation using water/$OE_2imC_3C$ (90/10, v/w). (n = 3, experimentally triplicate) Commercial is Culture Sure freezing medium (Fujifilm Wako Pure Chemical Corporation). Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

**Dead cell rate**

The toxicity of $OE_2imC_3C$ was examined for each cell type to confirm toxicity to cells, which is important as a freezing medium. The cells were recovered from the dish with trypsin treatment, centrifuged, and then immersed in water/$OE_2imC_3C$ (90/10, w/v) for 60 minutes to measure the dead cells rate. For all cells, the dead cell ratio in water/$OE_2imC_3C$ (90/10, w/v) was higher than that in PBS, but there was no toxicity that directly linked to the low viability (Fig. 2-16, 2-17). In the case of hNF, MDA, and PC9 cells with high viability, the dead cell ratio in water/$OE_2imC_3C$ (90/10, w/v) were lower than that in commercial. In contrast, mNF, BOSC, and WM cells, with low viability, the dead cell ratio in water/$OE_2imC_3C$ (90/10, w/v) were higher than the commercial (Maximum cell death rate was 35% for WM at room temperature and 16% for mNF at 0 °C.). The dead cell ratio in water/$OE_2imC_3C$ (90/10, w/v) at 0 °C was lower than at room temperature. A relationship was suggested between the toxicity of freezing medium to cells and viability. However, the dead cell rate in the commercial, which was considered to be highly toxic, was lower than that in PBS, which was

considered to be less toxic. Therefore, the toxicity to cells is unclear and requires further discussion. The poorly cryopreserved cell lines showed a high cell death rate even in PBS (Fig. 2-16). This means that poorly cryopreserved cell lines were very weak in suspended suspension.

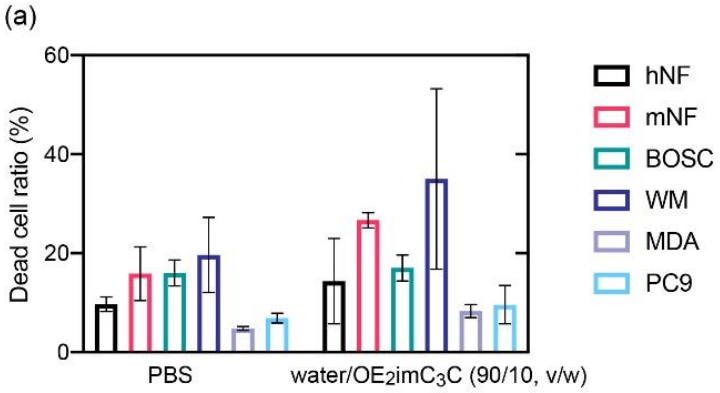



Fig. 2-16 Dead cell ratio after 60 min of immersion in water/$OE_2imC_3C$ (90/10, w/v) at room temperature. These cells were immersed as floating cells in water/$OE_2imC_3C$ (90/10, w/v) after trypsin treatment. (n = 3, biologically triplicate, one-way ANOVA) Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

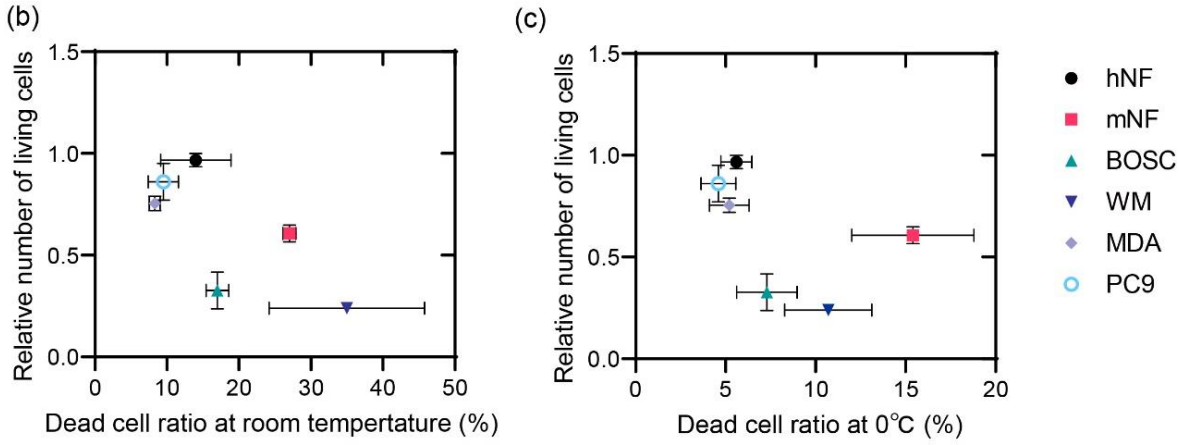



Fig. 2-17 The relation between the relative number of living hNF, mNF, BOSC, WM, MDA and PC9 cells and dead cell ratio at room temperature and 0 °C after 60 min of immersion in water/$OE_2imC_3C$ (90/10, w/v). These cells were immersed as floating cells in water/$OE_2imC_3C$ (90/10, w/v) after trypsin treatment. (n = 3, biologically triplicate, one-way ANOVA) Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

**Cell volume**

Non-cell-permeable cryoprotectant such as $OE_2imC_3C$ can inhibit extracellular ice crystal

formation and indirectly suppress intracellular ice crystal formation by intracellular dehydration. According to past literature, suppress intracellular ice crystal formation is more important than extracellular ice crystal formation[21, 24]. The degree of intracellular dehydration was investigated for each cell by measuring the cell volume after immersion in the freezing medium for 5 minutes. The cell volume in PBS was taken as 100. All cells had smaller volumes in water/$OE_2imC_3C$ (90/10, w/v) compared to PBS. hNF, MDA, and PC9 cells, which had high viability, cell volumes became so small that the intracellular dehydration in water/$OE_2imC_3C$ (90/10, w/v) was sufficient (Fig. 2-18). In contrast, mNF, BOSC, and WM cells, which had low viability, had large cell volumes in water/$OE_2imC_3C$ (90/10, w/v). These different dehydration behaviors were not based on the original cell size (Fig. 2-19). The relative cell volumes in water/$OE_2imC_3C$ (90/10, w/v) at 0 °C was lower than at room temperature. Therefore, it was suggested that the reason for the low viability may be insufficient intracellular dehydration. Commercial containing DMSO, a cell-permeable cryoprotectant, can suppress intracellular ice crystal formation by cell-permeable DMSO, but $OE_2imC_3C$, a non-cell-permeable cryoprotectant, can suppress intracellular ice crystal formation only by intracellular dehydration. For mNF, BOSC, and WM cells, which tend to have less intracellular dehydration, ice crystals may have formed intracellularly. These results suggest that intracellular dehydration with $OE_2imC_3C$ alone may not be able to completely suppress intracellular ice crystal formation, depending on the cell type, leading to a low viability.

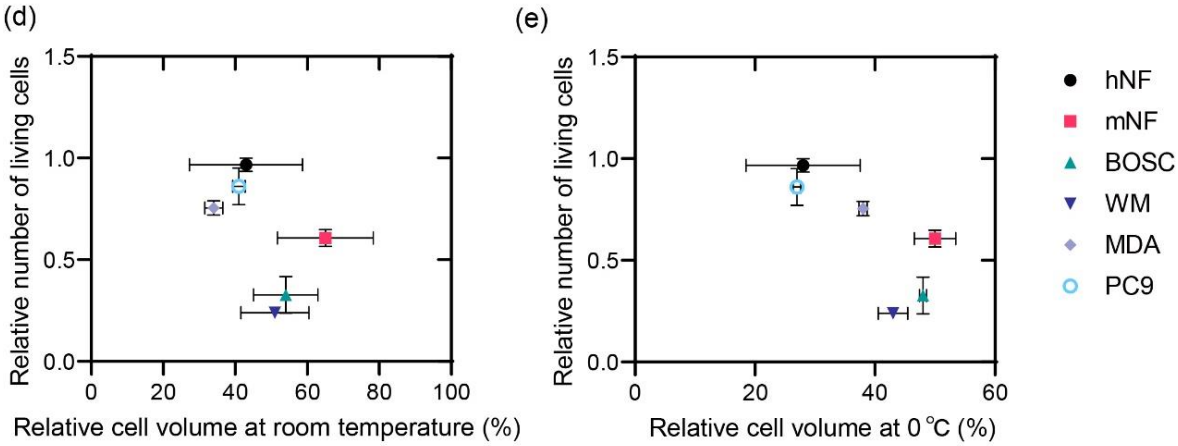



Fig. 2-18 The relation between the relative number of living hNF, mNF, BOSC, WM, MDA and PC9 cells and relative cell volumes at room temperature and 0 °C after 5 min of immersion in water/$OE_2imC_3C$ (90/10, w/v) (cell volumes at the same temperature in PBS were standardized as 100%). These cells were immersed as floating cells in water/$OE_2imC_3C$ (90/10, w/v) after trypsin treatment. (n = 3, biologically triplicate, one-way ANOVA) Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

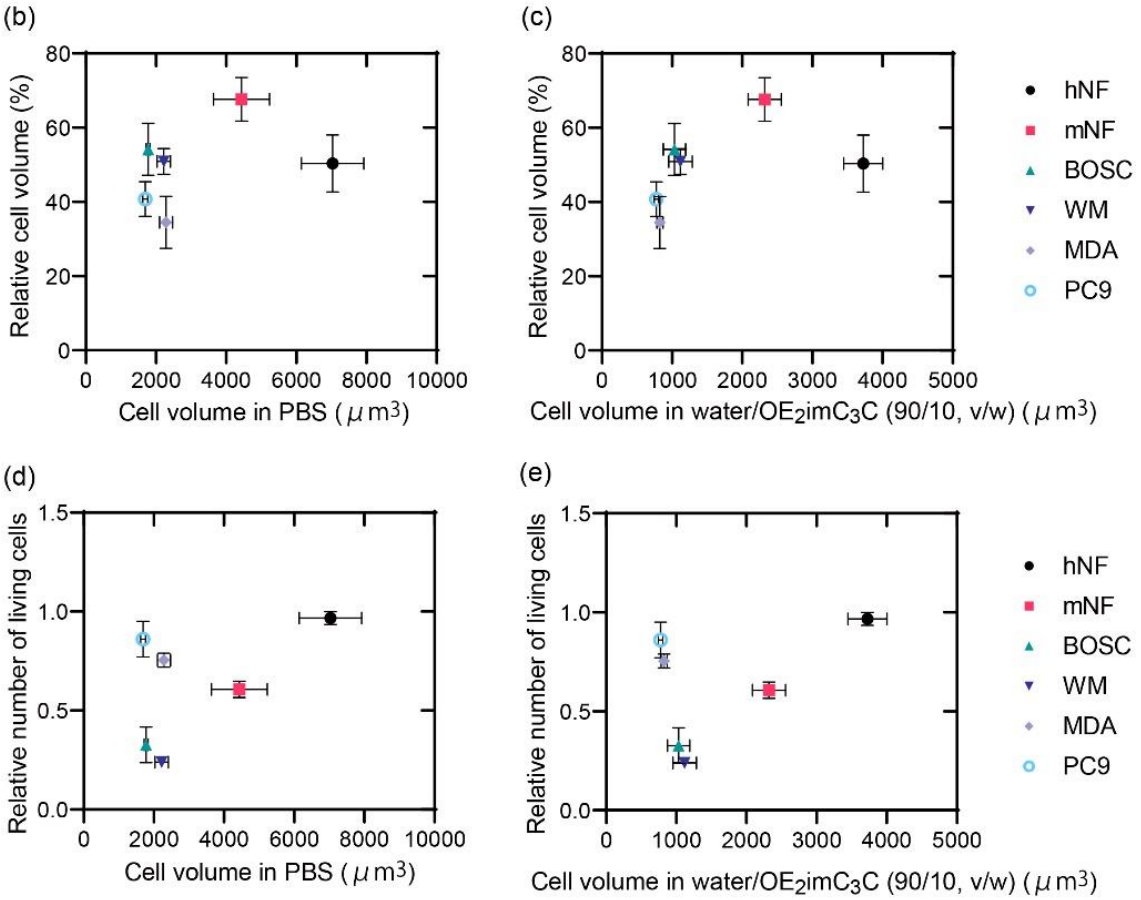



Fig. 2-19 The relation between relative cell volume after 5 min of immersion in water/$OE_2imC_3C$ (90/10, v/w) and absolute cell volume in PBS and water/$OE_2imC_3C$ (90/10, v/w) after 5 min of immersion. The relation between the relative number of living cells and absolute cell volume in PBS and water/$OE_2imC_3C$ (90/10, v/w) after 5 min of immersion. These cells were immersed as floating cells in water/$OE_2imC_3C$ (90/10, w/v) after trypsin treatment. (n = 3, biologically triplicate, one-way ANOVA) Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

**Water content**

If dehydration is insufficient, it is predicted that water remain inside the cells. I investigated the intracellular water content after dehydration depending on the cell type. The volume of the cells after dehydration was measured by adding a water/$OE_2imC_3C$ (90/10, w/v) and standing it for 5 minutes at room temperature, and the water content of the cells in the water/$OE_2imC_3C$ (90/10, w/v) was calculated from the weight of the cells in the water/$OE_2imC_3C$ (90/10, w/v) and the weight of the cells after vacuum drying. As a result of examining the intracellular water content, hNF, MDA, and PC9 cells, which had high viability, had a low intracellular water content in water/$OE_2imC_3C$ (90/10, w/v).

In contrast, mNF, BOSC, and WM cells, which had low viability, had high intracellular water content in water/$OE_2imC_3C$ (90/10, w/v) (Fig. 2-20). The intracellular water content in water/$OE_2imC_3C$ (90/10, w/v) at 0 °C was lower than at room temperature, but the decrease was small for cells with low viability. This different behavior may also be based on the original intracellular water content (Fig. 2-21). In other words, I found a clear relationship between the water content of cells in water/$OE_2imC_3C$ (90/10, w/v) and the cell viability. Therefore, the reason why the cryoprotecting effect is low is that dehydration of the cells is insufficient, and intracellular water becomes ice crystals, resulting in toxicity.

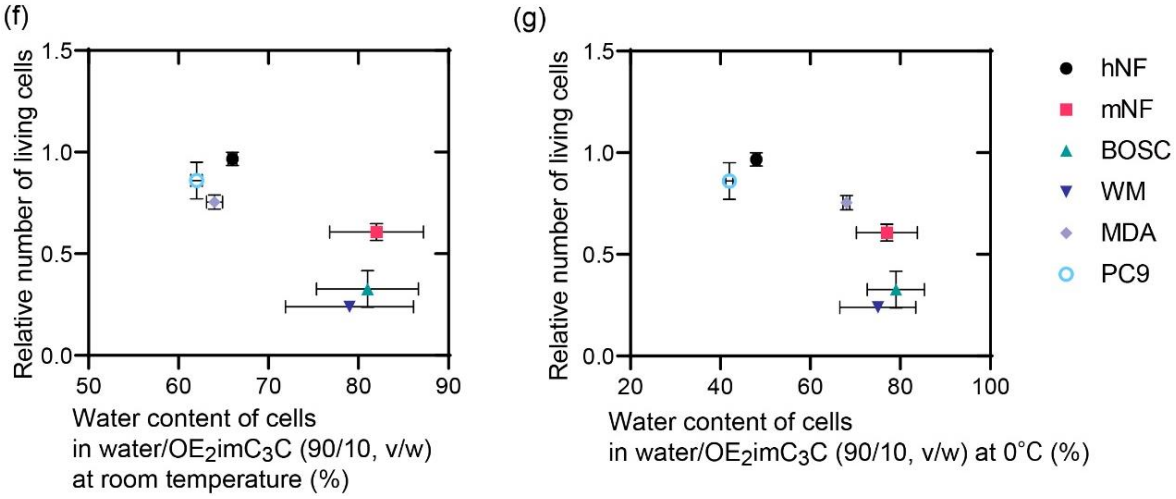



Fig. 2-20 The relation between the relative numbers of living hNF, mNF, BOSC, WM, MDA and PC9 cells and the water contents of cells in water/$OE_2imC_3C$ (90/10, v/w) at room temperature and 0 °C. These cells were immersed as floating cells in water/$OE_2imC_3C$ (90/10, w/v) after trypsin treatment. (n = 3, biologically triplicate, one-way ANOVA) Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

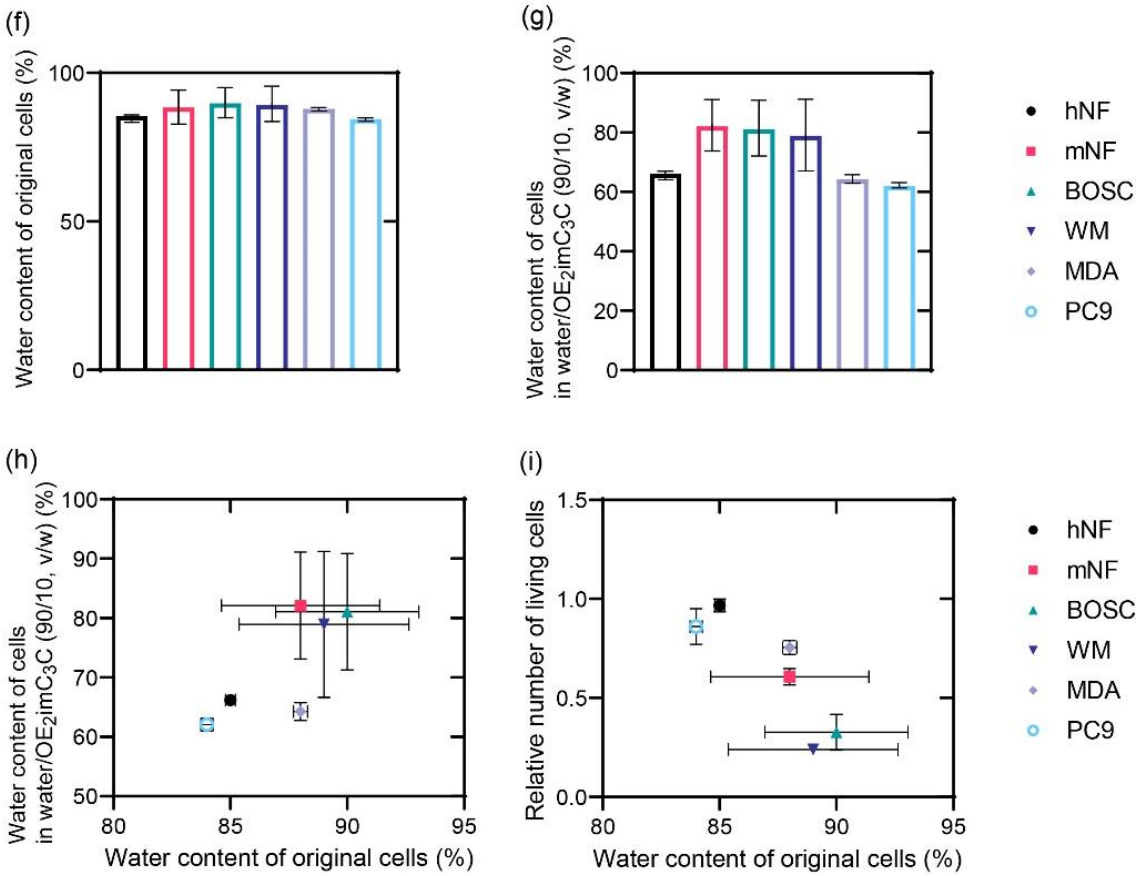



Fig. 2-21 Water content of original cells in PBS and cells after dehydrated in water/$OE_2imC_3C$ (90/10, v/w). The relation between water content of original cells and cells after dehydrated in water/$OE_2imC_3C$ (90/10, v/w). The relation between the relative number of living cells and water content of cells dehydrated in water/$OE_2imC_3C$ (90/10, v/w). These cells were immersed as floating cells in water/$OE_2imC_3C$ (90/10, w/v) after trypsin treatment. (n = 3, biologically triplicate, one-way ANOVA) Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

These findings highlighted the difficulty of achieving sufficient dehydration of all cells using $OE_2imC_3C$ only.

## 2.8 Optimal structure of zwitterions as cryoprotectants.

**Comparison with ionic liquid**

Free ions called ionic liquids, are known to be toxic[8, 9, 12, 25]. I compared the cryoprotecting effects of zwitterion and ionic liquid. mNF and BOSC were cryopreserved using ionic liquids, similar non-zwitterionic-type ions. [$OE_2$eim]OAc, which has a very similar structure to $OE_2imC_3C$, was used as the ionic liquid. Surprisingly, in the case of BOSC, toxic ionic liquids[6] also exerted some

cryoprotecting effect (Fig. 2-22), indicating that toxicity at room temperature may not be related to cryoprotecting effect. The findings also mean that toxic compounds may be potential cryoprotectant candidates.

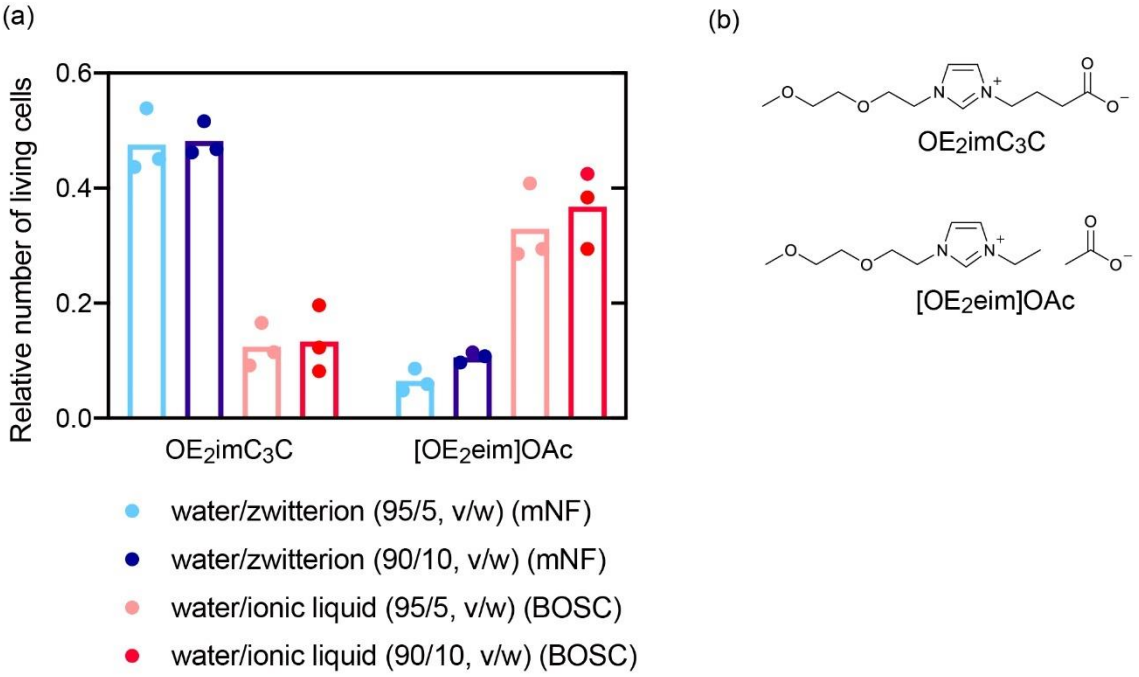



Fig. 2-22 The relative number of living mNF and BOSC cells after cryopreservation using 5% and 10% (w/v) [$OE_2$eim]OAc and $OE_2imC_3C$ aqueous solutions. The structures of $OE_2imC_3C$ and [$OE_2$eim]OAc. (n = 3, experimentally triplicate) Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

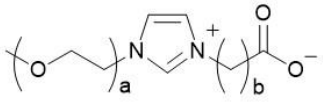
**Comparison of zwitterion structures**

In the above experiments, only $OE_2imC_3C$ aqueous solution has been used as freezing medium. On the other hand, other aprotic zwitterions also have positive and negative charges and can interact strongly with water, so they are expected to function as cryoprotectant. Therefore, using mNF and BOSC, which had a low viability after cryopreservation using $OE_2imC_3C$ aqueous solution, I investigated which zwitterion is the best cryoprotectant. Various 5, 10% (w/v) zwitterion (Fig. 2-23 for structures) aqueous solutions were used as freezing media. As a result, the cryoprotecting effect differed greatly for each zwitterion (Fig. 2-24). For mNF, the zwitterion aqueous solution that showed the highest cryoprotecting effect was water/$VimC_4S$ (90/10, w/v), with the relative number of living cells of 0.63. This was higher than the relative number of living cells of 0.60 when water/$OE_2imC_3C$ (90/10, w/v) was used. In addition, when using $C_1imC_3C$ and $VimC_3S$ aqueous solution, the relative number of living cells close to 0.60 was obtained. For BOSC, the zwitterion aqueous solution that showed the highest cryoprotecting effect was water/$VimC_3S$ (95/5, w/v), with the relative number of

living cells of 0.50. This was higher than the relative number of living cells of 0.37 when water/$OE_2imC_3C$ (90/10, w/v) was used. In addition, when using $VimC_4S$ and $AimC_4S$ aqueous solution, the relative number of living cells close to 0.40 was obtained. Thus, cell viability after cryopreservation using zwitterion was found to be dependent on both zwitterion species and cell types. Among these results, water/$OE_2imC_3C$ (90/10, w/v), water/$VimC_3S$ (90/10, w/v), water/$C_1imC_3C$ (95/5, w/v), water/$C_1imC_5C$ (90/10, w/v), water/$C_1imC_2C$ (90/10, w/v) and water/$PyC_3C$ (90/10, w/v) were the freezing media that showed relatively good results for both mNF and BOSC. For example, when water/$VimC_4S$ (90/10, w/v), a zwitterion with a high viability, was used, the relative number of living cells of mNF and BOSC were 0.63 and 0.45, respectively. Conversely, the zwitterion that showed the lowest viability was water/$OE_2imC_3S$ (95/5, w/v), and the relative number of living cells of mNF and BOSC were 0.10 and 0.047, respectively. Thus, different zwitterion species had different cryoprotecting effects. A direct relationship between cryoprotecting effect and structure of zwitterions was explored, but no clear relationship was found.

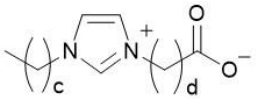
DMSO

Cation:imidazolium Anion: carboxylate



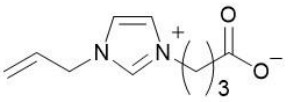
$OE_aimC_bC$

a = 1 or 2
b = 3 or 5

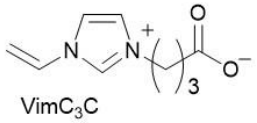


$C_{c-1}imC_dC$

c = 1 or 4
d = 2, 3 or 5

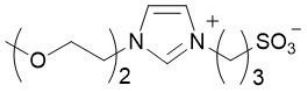


$AimC_3C$

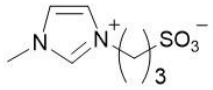


$VimC_3C$

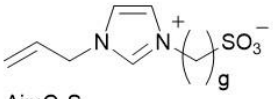
Cation:imidazolium Anion: sulfonate



$OE_2imC_3S$

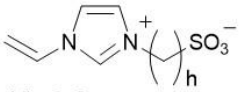


$C_1imC_3S$



$AimC_gS$

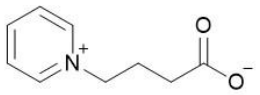
g = 3 or 4



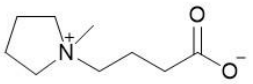
$VimC_nS$

h = 3 or 4

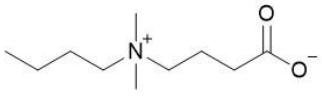
Cation: others Anion: carboxylate



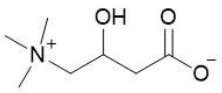
$PyC_3C$



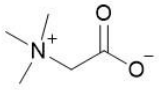
$PyrrC_3C$



$N_{1,1,4}C_3C$



L-carnitine



trimethylglycine

Fig. 2-23 The structures of the zwitterions used in this study. They are categorized by the cation and anion species. Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

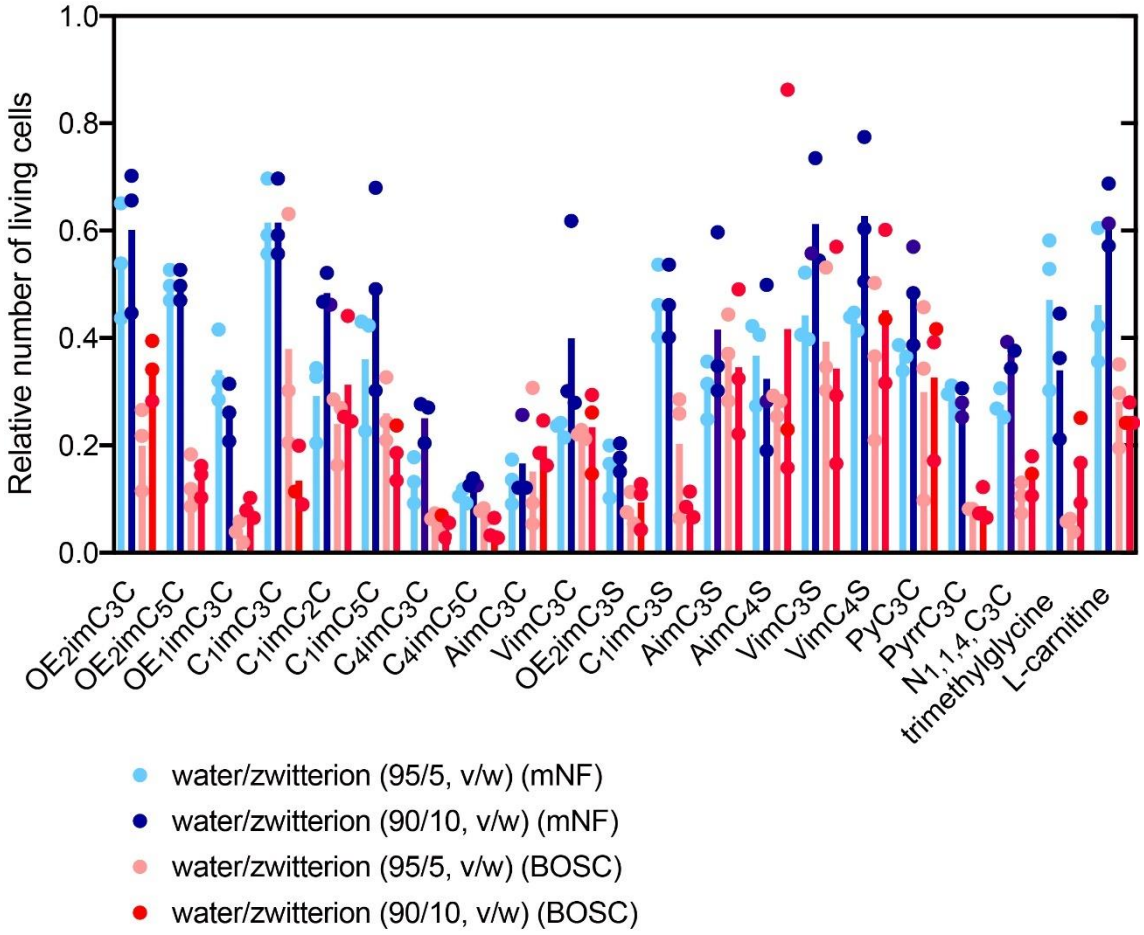



Fig. 2-24 The relative number of living mNF and BOSC cells after cryopreservation using water/zwitterion (95/5 and 90/10, v/w). (n = 3, experimentally triplicate) Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

**Osmotic pressure**

I investigated the relationship between the cryoprotecting effect and the osmotic pressure of zwitterion aqueous solution. When the osmotic pressure of water/zwitterion (90/10, w/v) and the molar concentration of zwitterion were plotted, a proportional relationship was observed although there was some variation (Fig. 2-25). From this, it was found that zwitterion increases the osmotic pressure in almost proportion to the concentration, regardless of its molecular structure. This trend was followed by the high concentration 25 and 35% (w/v) $OE_2imC_3C$ aqueous solutions.

When the osmotic pressure of each zwitterion aqueous solution was plotted against the relative number of living mNF and BOSC cells, no clear correlation was found (Fig. 2-26). This indicated that osmotic pressure was not the dominant factor in cryopreservation with zwitterion. Increasing the osmotic pressure and promoting intracellular dehydration lead to suppression of intracellular ice crystal formation, but no clear trend was observed in this study. This is because, as mentioned above, once the osmotic pressure reaches a certain level, cell contraction reaches a limit and the cell volume does not change (Fig. 2-13). However, the shrinkage limit alone cannot fully explain the phenomenon, and other causes are thought to be involved.

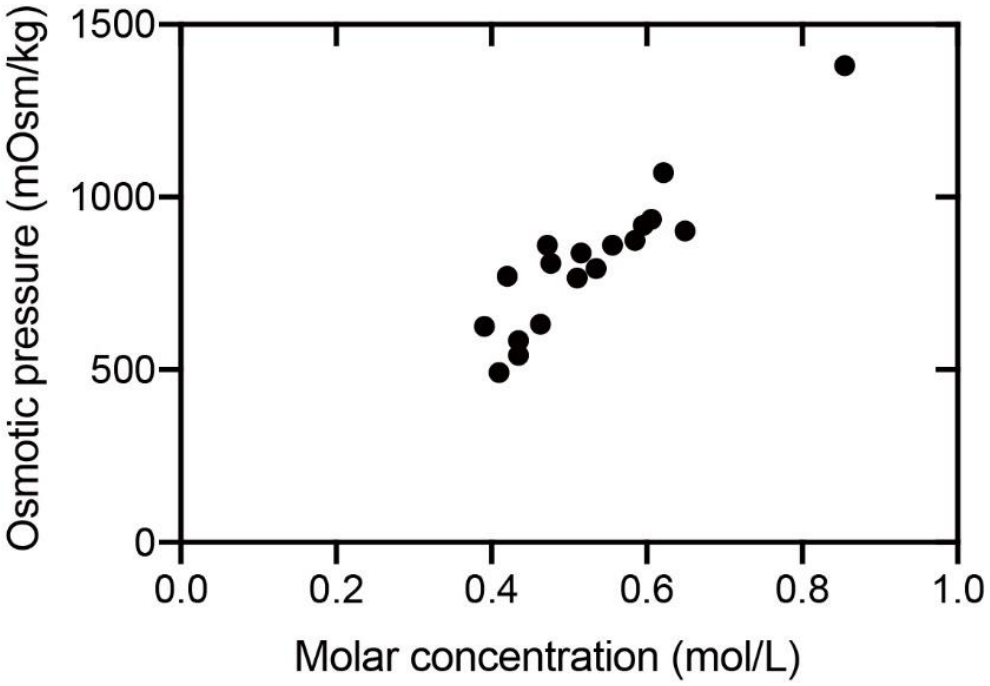



Fig. 2-25 The relationship between osmotic pressure and molar concentration of water/zwitterion (90/10, v/w). Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

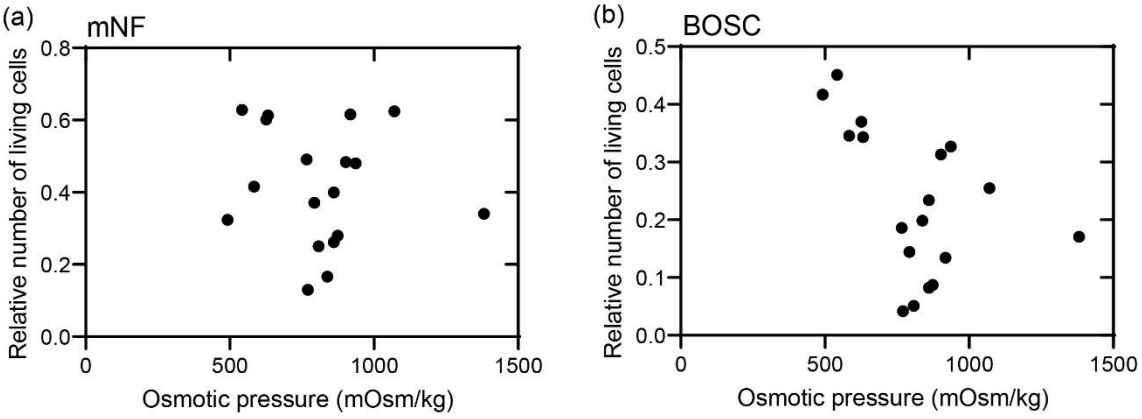


Fig. 2-26 The relationship between osmotic pressure of water/zwitterion (90/10, v/w) and the relative number of living mNF and BOSC cells after cryopreservation in the zwitterion aqueous solutions. Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

**Glass transition temperature**

The state of each water/zwitterion (90/10, w/v) at low temperature was investigated using differential scanning calorimetry (DSC). This time, I set the following temperature conditions and measured the glass transition temperature using DSC-60A plus (Shimadzu Corporation) (Fig. 2-27).

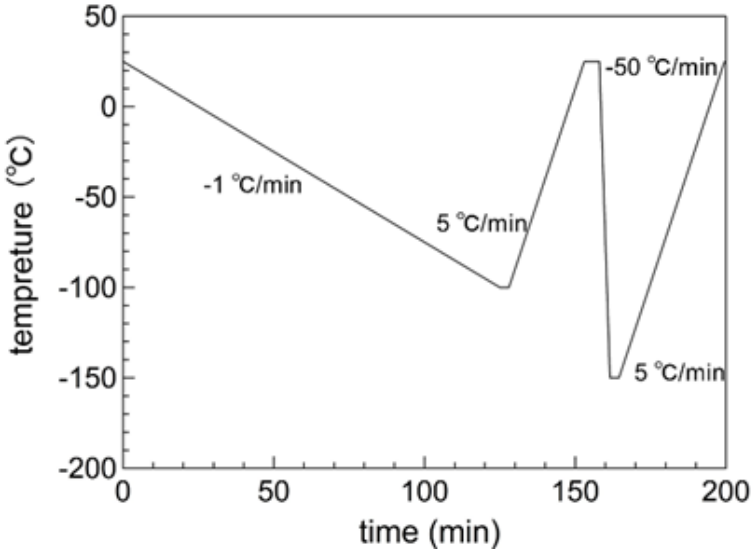



Fig. 2-27 Temperature setting of DSC

Organic ions such as zwitterion are highly viscous due to their strong electrostatic interactions, and tend to vitrify[8, 21, 26]. All zwitterion aqueous solutions except $VimC_4S$ were confirmed to vitrify (Table 2-1).

Table 2-1 The glass transition temperature and the proportion of unfrozen water of water/zwitterion (90/10, v/w), and the relative number of living mNF and BOSC cells after cryopreservation in the indicated solutions. Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

| | Glass transition temperature $T_g$ (°C) | Unfrozen water proportion (wt%) | Relative number of living mNF cells | Relative number of living BOSC cells |
|---|---|---|---|---|
| 10% (w/v) $OE_2imC_3C$ | -85.69 | 17 | 0.60 | 0.37 |

| | | | | |
|---|---|---|---|---|
| 10% (w/v) $OE_2imC_5C$ | -83.34 | 19 | 0.50 | 0.14 |
| 10% (w/v) $OE_1imC_3C$ | -86.07 | 21 | 0.26 | 0.082 |
| 10% (w/v) $C_1imC_3C$ | -95.22 | 16 | 0.62 | 0.13 |
| 10% (w/v) $C_1imC_2C$ | -96.61 | 18 | 0.48 | 0.31 |
| 10% (w/v) $C_1imC_5C$ | -84.51 | 29 | 0.49 | 0.19 |
| 10% (w/v) $C_4imC_3C$ | -75.30 | 17 | 0.25 | 0.050 |
| 10% (w/v) $C_4imC_5C$ | -74.59 | 23 | 0.13 | 0.042 |
| 10% (w/v) $AimC_3C$ | -85.00 | 21 | 0.17 | 0.20 |
| 10% (w/v) $VimC_3C$ | -85.74 | 24 | 0.40 | 0.23 |
| 10% (w/v) $OE_2imC_3S$ | -85.48 | 20 | 0.18 | 0.090 |
| 10% (w/v) $C_1imC_3S$ | -88.88 | 27 | 0.47 | 0.090 |
| 10% (w/v) $AimC_3S$ | -85.76 | 11 | 0.42 | 0.35 |
| 10% (w/v) $AimC_4S$ | -86.07 | 12 | 0.32 | 0.42 |
| 10% (w/v) $VimC_3S$ | -81.06 | 28 | 0.61 | 0.34 |
| 10% (w/v) $VimC_4S$ | -[a] | 10 | 0.63 | 0.45 |
| 10% (w/v) $PyC_3C$ | -90.35 | 23 | 0.48 | 0.33 |
| 10% (w/v) $PyrrC_3C$ | -91.85 | 21 | 0.28 | 0.087 |
| 10% (w/v) $N_{1,1,4,}C_3C$ | -74.96 | 23 | 0.37 | 0.14 |
| 10% (w/v) trimethylglycine | -105.09 | 27 | 0.34 | 0.17 |
| 10% (w/v) L-carnitine | -85.84 | 25 | 0.62 | 0.25 |
| [a]not detected over -100 °C | | | | |

I investigated the relationship between the cryoprotecting effect and the vitrification of zwitterion aqueous solution. The glass transition temperature of zwitterion was concentrated around -85 °C, which is the temperature during cryopreservation. Since it can be stored without ice crystals in a vitrified state, it was thought that vitrification would have a high viability. However, some zwitterions had glass transition temperatures higher than -85 °C and some zwitterions had lower than -85 °C, but no relationship with the relative number of living cells was observed (Fig. 2-28). In addition, the relative number of living cells when using non-vitrified water/$VimC_4S$ (90/10, w/v) was as high as 0.66 and 0.35 for mNF and BOSC, respectively. That is, there was no correlation between vitrification and cell viability.

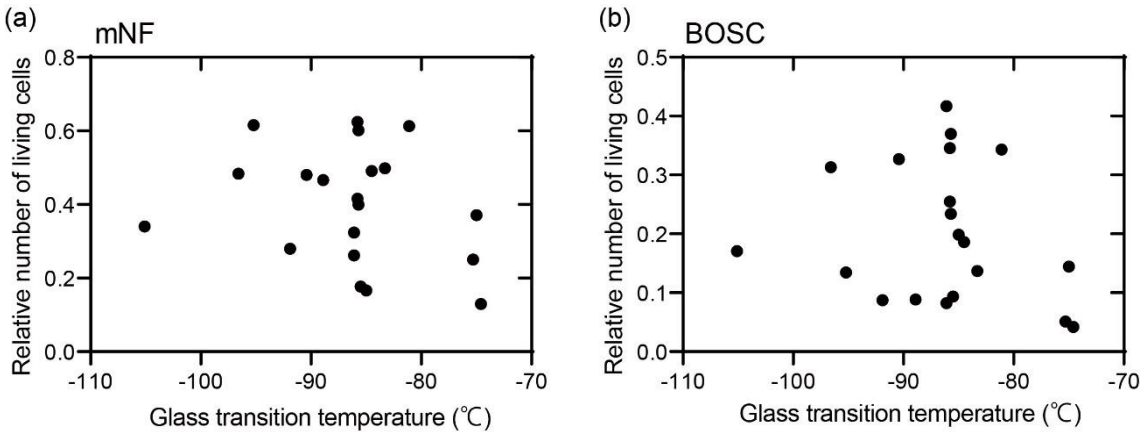



Fig. 2-28 The relation between glass transition temperature of water/zwitterion (90/10, v/w) and the relative number of living mNF and BOSC cells after cryopreservation in the zwitterion aqueous solutions. Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

To confirm that vitrification is not directly related to the cell viability, hNF cells were cryopreserved at -85 °C, which is the glass transition temperature of water/$OE_2imC_3C$ (90/10, w/v), and -80 °C, which is before the glass transition temperature of water/$OE_2imC_3C$ (90/10, w/v). There was no significant difference in the cell viability of hNF cell depending on the freezing temperature (Fig. 2-29). Therefore, there is no relationship between vitrification and cell viability.

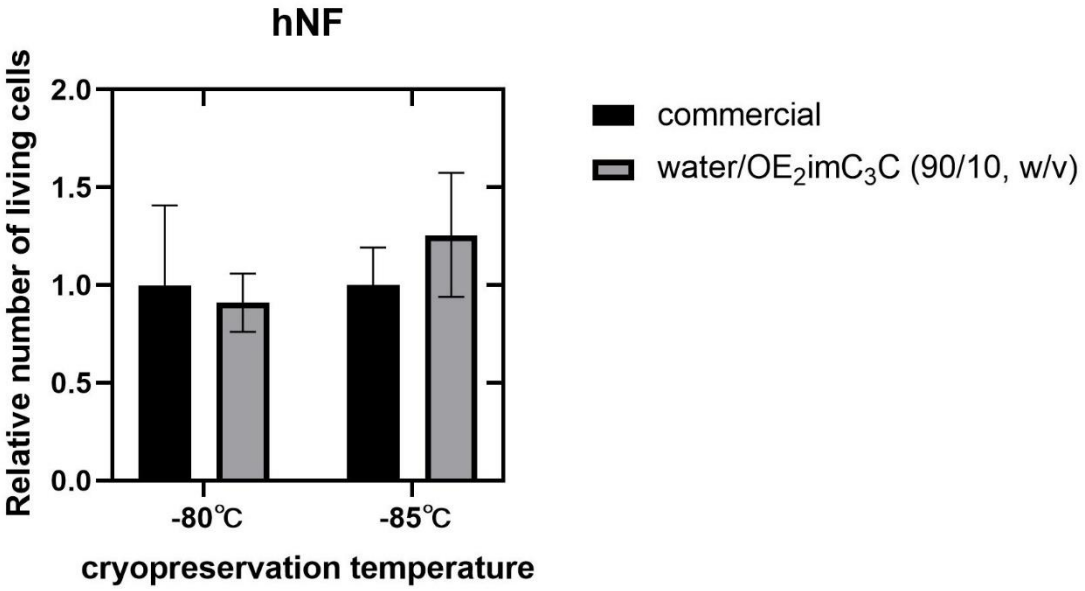



Fig. 2-29 The relative number of living hNF cells after cryopreservation using water/$OE_2imC_3C$ (90/10, w/v) at -85 °C, which is the glass transition temperature, and -80 °C, which is before the glass transition temperature. (n = 3, biologically triplicate, one-way ANOVA) There is no statistical significance

between the samples ($p > 0.1$)

**The proportion of unfrozen water**

I investigated the relationship between the cryoprotecting effect and the proportion of unfrozen water of zwitterion aqueous solution. As a result, even if the proportion of unfrozen water was large, some had high viability while others had low viability. Smaller molecular weight zwitterion aqueous solutions showed higher proportion of unfrozen water due to their higher molarity. Sulfonate-based zwitterion aqueous solutions showed a higher proportion of unfrozen water than carboxylate-based zwitterion aqueous solutions. However, there was no correlation between the proportion of unfrozen water in the zwitterion aqueous solution and the relative number of living cells (Fig. 2-30). Therefore, unfrozen water may be required to protect cells from extracellular ice crystals. However, excessive amounts of unfrozen water may not provide additional cryoprotectants. These findings suggest that the type and concentration of zwitterions greatly affect cell viability after cryopreservation.

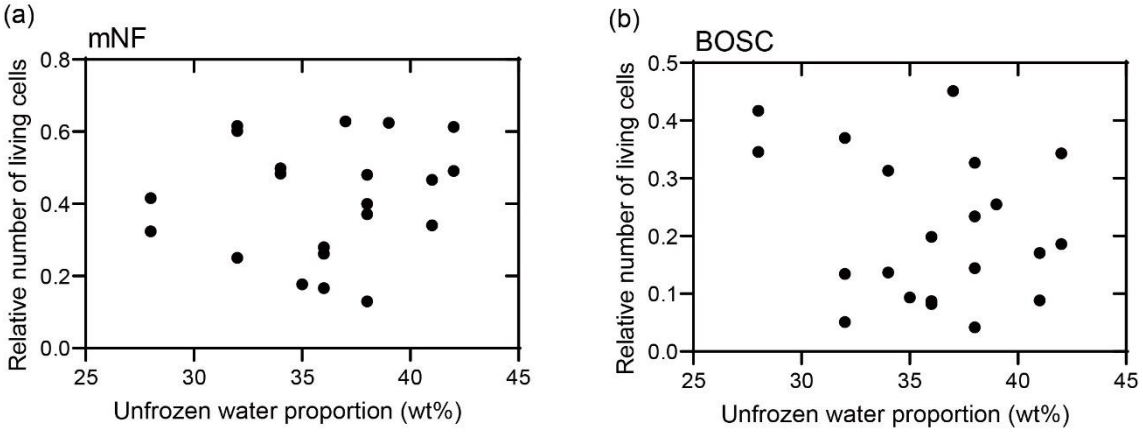



Fig. 2-30 The relation between the proportion of unfrozen water of water/zwitterion (90/10, v/w) and the relative number of living mNF and BOSC cells after cryopreservation in the zwitterion aqueous solutions. Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

From these results, although vitrification, osmotic pressure, and the proportion of unfrozen water differed depending on the structure and concentration of zwitterion, no clear correlation with cell viability was found. Even if the structure and concentration of zwitterion were changed, the cell viability could not be made equal to that using commercial. Therefore, it was suggested that vitrification, osmotic pressure, and the proportion of unfrozen water are not the main factors for cryoprotectant, because simply inhibiting ice crystal formation does not increase the cell viability. It was not possible to prepare a general-purpose and effective freezing medium only with zwitterion. From now on, the key factors of efficient cryopreservation with zwitterion need to be investigated.

2.9 Blending with non-cell-permeable cryoprotectants

**Combination with zwitterion**

I also investigated the mixing of different types of zwitterions. $OE_2imC_3C$ and zwitterion with high viability were mixed, and mNF and BOSC cells were cryopreserved. The relative number of living cells when using water/$OE_2imC_3C$ (90/10, w/v) was 0.64 and 0.19 for mNF and BOSC (Fig. 2-31). In the case of mNF cells, the cell viability was highest when using water/$OE_2imC_3C$/L-carnitine (80/10/10, w/w/v) (the relative number of living cells: 0.79). In the case of BOSC cells, the cell viability was highest when using water/$OE_2imC_3C$/$AimC_4S$ (80/10/10, w/w/v) (the relative number of living cells: 0.23). Therefore, there was no significant difference in the cell viability when using the zwitterion mixtures compared to when using $OE_2imC_3C$ alone. Moreover, the cell viability was lower than that using commercial when using any of the zwitterion mixtures. There is no significant synergistic cryoprotecting effect when zwitterions are combined.

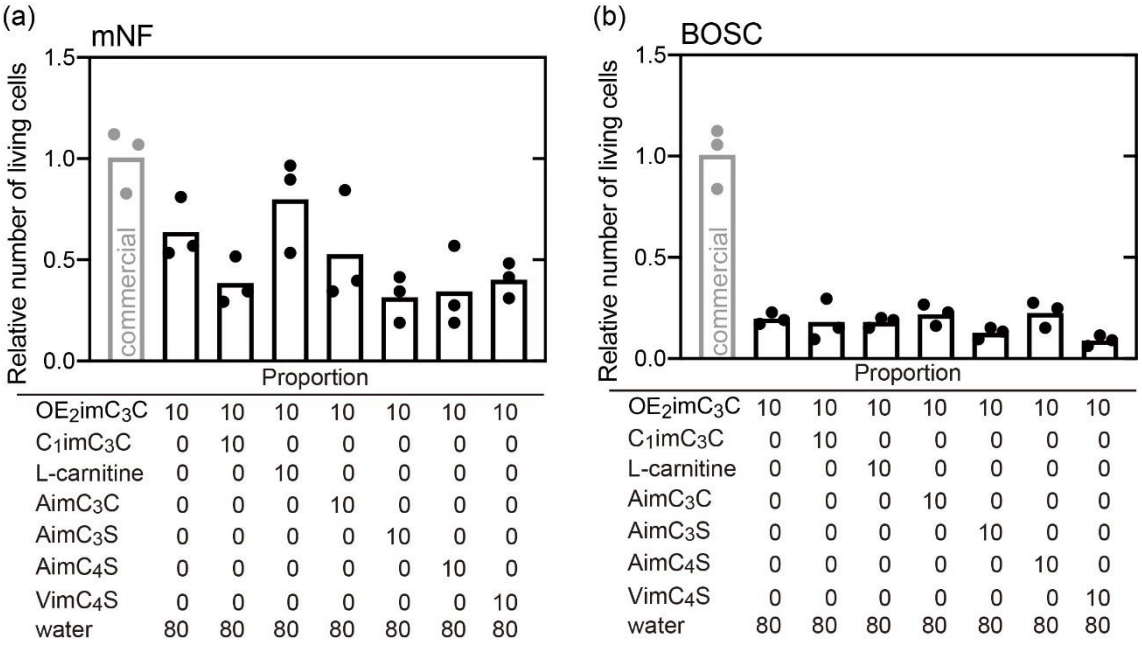



Fig. 2-31 The relative number of living mNF and BOSC cells after cryopreservation with water/zwitterion/zwitterion (80/10/10, v/w/w). (n = 3, experimentally triplicate) Commercial is Culture Sure freezing medium (Fujifilm Wako Pure Chemical Corporation). Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

**Combination with non-cell-permeable cryoprotectants**

Sucrose and FBS, which are typical non-cell-permeable cryoprotectants, were added to water/$OE_2imC_3C$ (90/10, w/v), and mNF and BOSC cells were cryopreserved. When using samples containing these non-cell-permeable cryoprotectants, there was no significant change in the relative

number of living cells compared to using water/$OE_2imC_3C$ (90/10, w/v) alone (Fig. 2-32). As expected, it was confirmed that $OE_2imC_3C$ has a strong ability to inhibit ice crystal formation outside the cell, while its ability to inhibit ice crystal formation inside the cell is low.

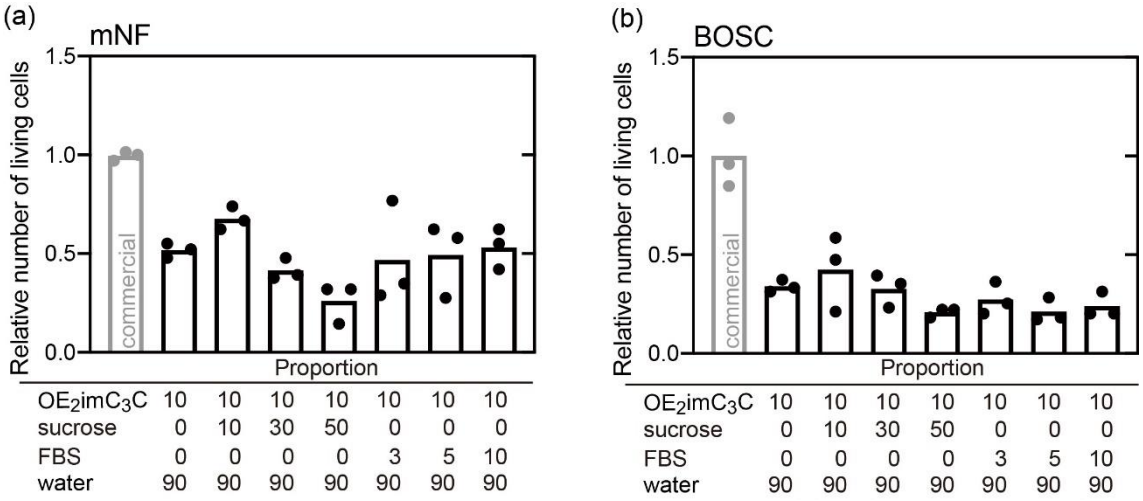



Fig. 2-32 The relative number of living mNF and BOSC cells after cryopreservation with water/$OE_2imC_3C$ (90/10, v/w) mixed non-cell-permeable additives (sucrose and FBS). Total compositions in some cases are over 100 to be clear. (n = 3, experimentally triplicate) Commercial is Culture Sure freezing medium (Fujifilm Wako Pure Chemical Corporation). Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

## 2.10 Blending with cell-permeable cryoprotectants

The reason why zwitterion alone does not provide a higher viability than that using commercial is considered to be that non-cell-permeable cryoprotectant basically cannot strongly suppress intracellular ice crystal formation. Here, when sucrose, which is a typical non-cell-permeable cryoprotectant, and DMSO and glycerol, which are cell-permeable cryoprotectant were compared in the proportion of unfrozen water at equimolar concentrations (0.6 mol%), the proportion of unfrozen water was 11 wt% for the sucrose aqueous solution, 14, 15 wt% for the DMSO and glycerol aqueous solution, and 31 wt% for the $OE_2imC_3C$ aqueous solution. Since zwitterion has an ability to inhibit extracellular ice crystal formation that surpasses that of sucrose, it is expected to become a very good freezing medium by adding a substance that inhibits intracellular ice crystal formation.

DMSO and glycerol, which are cell-permeable cryoprotectants, were added to water/$OE_2imC_3C$ (90/10, w/v). As a result, the relative number of living cells was higher when using samples containing these cell-permeable cryoprotectants than when using water/$OE_2imC_3C$ (90/10, w/v) alone (Fig. 2-33). The combination with the highest viability was water/$OE_2imC_3C$/DMSO (90/10/15, w/w/v). The relative number of living mNF and BOSC cells when using water/$OE_2imC_3C$ (90/10, w/v) were 0.51

and 0.32, whereas the relative number of living mNF and BOSC cells when using water/$OE_2imC_3C$/DMSO (90/10/15, w/w/v) were 1.13 and 1.14. Glycerol is known to be less permeable and more toxic than DMSO[21], corresponding to the lower relative number of living cells. Water/$OE_2imC_3C$/DMSO (90/10/15, w/w/v) showed the same cryoprotecting effect as the commercial. For example, the cryoprotecting effect of water/DMSO (90/10/10, w/v) was lower than that of water/$OE_2imC_3C$/DMSO (90/10/15, w/w/v), indicating the importance of combining the zwitterion and DMSO.

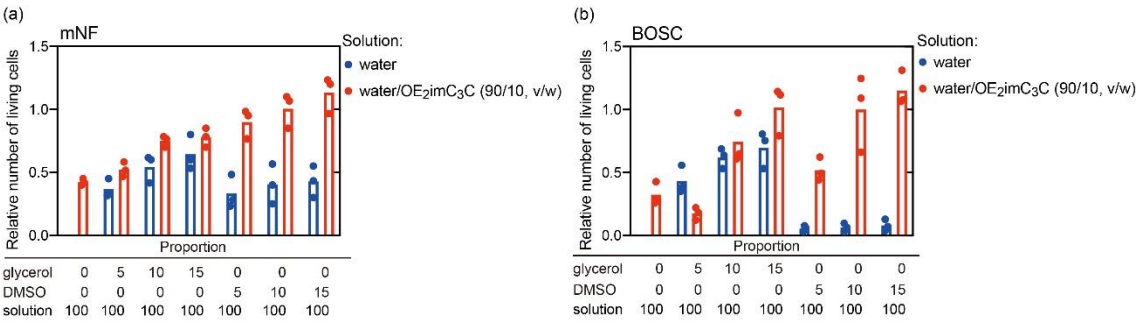



Fig. 2-33 The relative number of living mNF and BOSC cells after cryopreservation with water/$OE_2imC_3C$ (90/10, v/w) mixed cell-permeable additives (glycerol, DMSO). Total compositions in some cases are over 100 to be clear. (n = 3, experimentally triplicate) Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

In order to confirm whether any zwitterion has the same effect mixed with DMSO, the cells were cryopreserved using $OE_2imC_3C$, $VimC_3S$, trimethylglycine, L-carnitine,which had high viability with zwitterion alone, and $C_1imC_3S$, which had a low viability with zwitterion alone. The cells with low viability when using only zwitterion increased in the relative number of living cells when adding DMSO (Fig. 2-34). In particular, the relative number of living cells when using water/$OE_2imC_3C$/DMSO (90/10/15, w/w/v) or water/$C_1imC_3S$/DMSO (90/10/15, w/w/v) were 0.83, 0.92 for mNF, and 1.0, 1.01 for BOSC, which were equivalent to when using commercial.

Although zwitterions with various structures were investigated, the most suitable structure for cryoprotectant was not found. $OE_2imC_3C$ and $VimC_3S$ had a higher viability with zwitterion alone, but when combined with DMSO, $OE_2imC_3C$ had a large improvement in viability, while $VimC_3S$ had a small improvement in viability. $C_1imC_3S$ had a lower viability with zwitterion alone, but when combined with DMSO, the viability was greatly improved. In other words, the improvement in viability when combined with DMSO was not as significant as that of zwitterion alone.

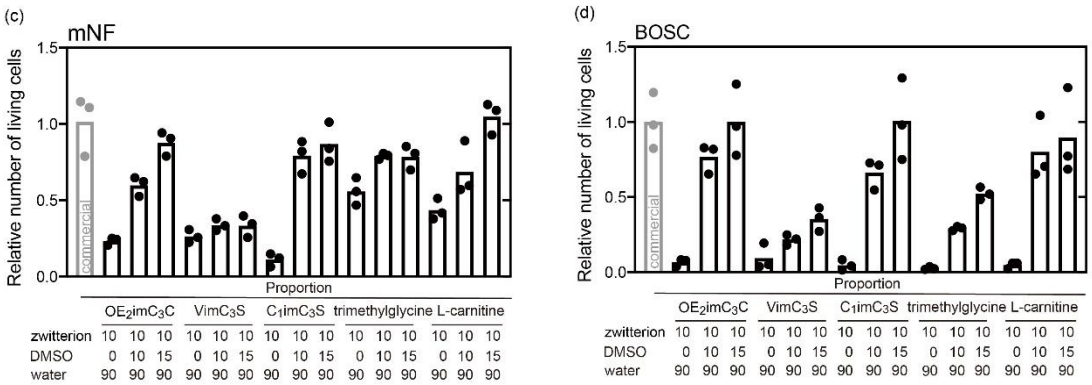



Fig. 2-34 The relative number of living mNF, BOSC cells after cryopreservation with mixtures of water/zwitterion (90/10, v/w) and DMSO. Total compositions in some cases are over 100 to be clear. (n = 3, experimentally triplicate) Commercial is Culture Sure freezing medium (Fujifilm Wako Pure Chemical Corporation). Material from: Kato, Y. *et al*., Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

In order to confirm whether other cells show the same tendency when this zwitterion/DMSO mixture is used, seven types of cells were cryopreserved using a zwitterion/DMSO mixture. The cells that showed a high viability with $OE_2imC_3C$ alone showed the equal to or higher viability than that with the commercial when using the mixture. The cells that showed a low viability with $OE_2imC_3C$ alone improved the viability when using the mixture to the extent the viability was equal to or higher than when using the commercial (Fig. 2-35).

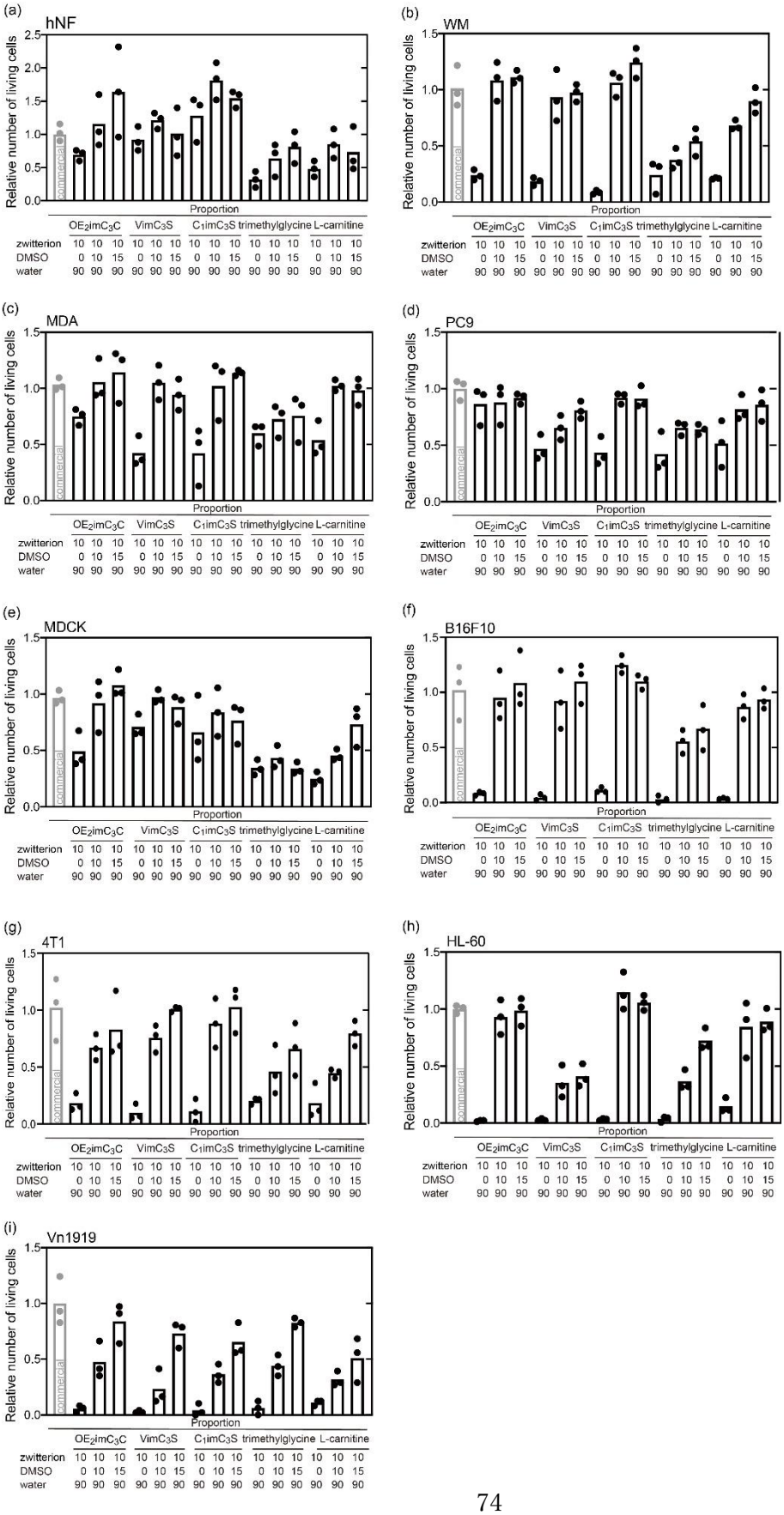

(a)
hNF
(b)
WM
(c)
MDA
(d)
PC9
(e)
MDCK
(f)
B16F10
(g)
4T1
(h)
HL-60
(i)
Vn1919
Relative number of living cells
commercial
Proportion
OE2imC3C
VimC3S
C1imC3S
trimethylglycine
L-carnitine
zwitterion
DMSO
water

Fig. 2-35 The relative number of living hNF, WM, MDA, PC9, MDCK, B16F10, 4T1, HL-60 and Vn1919 cells after cryopreservation with mixtures of water/zwitterion (90/10, v/w) and DMSO. (n = 3, experimentally triplicate) Commercial is Culture Sure freezing medium (Fujifilm Wako Pure Chemical Corporation). Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

In order to confirm whether the same cryoprotecting effect can be obtained even with cells that are vulnerable to freezing, K562 and OVMANA cells were cryopreserved using a mixture of zwitterion and DMSO. As expected, the cell viability was low when using commercial, but the cell viability increased when using a mixture of zwitterion and DMSO (Fig. 2-36). In particular, the relative number of living cells of K562 and OVMANA were 1.7, 1.8, 1.6, and 1.5 when water/$OE_2imC_3C$/DMSO (90/10/15, w/w/v) or water/$C_1imC_3S$/DMSO (90/10/15, w/w/v) was used for any cell, which was greatly improved compared to when using commercial.

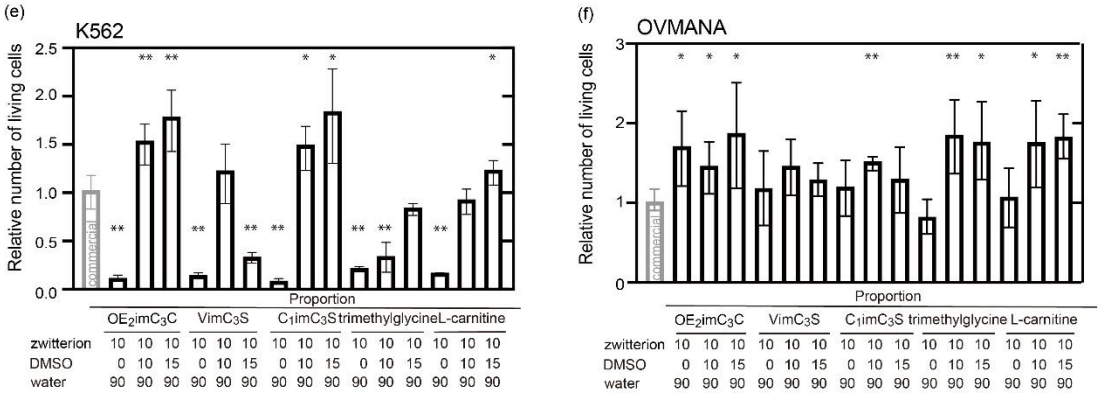



Fig. 2-36 The relative number of living K562, and OVMANA cells after cryopreservation with mixtures of water/zwitterion (90/10, v/w) and DMSO. (*: $p<0.1$, **: $p<0.05$, compared to commercial) Total compositions in some cases are over 100 to be clear. (n = 3, biologically triplicate, one-way ANOVA) Commercial is Culture Sure freezing medium (Fujifilm Wako Pure Chemical Corporation). Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

I investigated the proliferation rate after cryopreservation using a mixture of zwitterion and DMSO. In the case of K562, cells after cryopreserved using a mixture of zwitterion and DMSO proliferated at the same rate as cells after cryopreserved using a commercial (Fig. 2-37). However, in the case of OVMANA, although zwitterion alone sample showed a high cryoprotecting effect, the proliferation rate was very low.

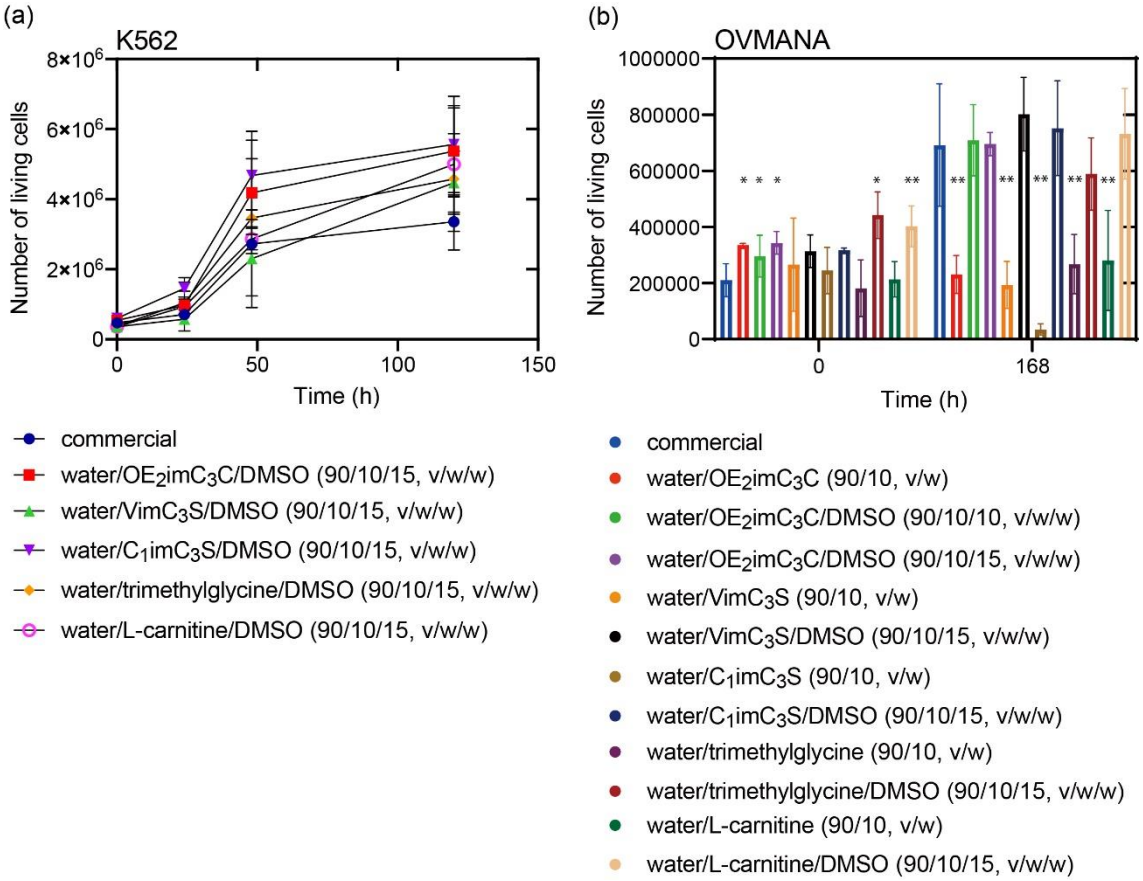



Fig. 2-37 Growth curves of K562 cells after cryopreservation with the indicated solutions. Number of living cells of OVMANA at 0 and 168 h after freezing and thawing with the indicated solutions (*p <0.1, **p <0.05). (n = 3, biologically triplicate, one-way ANOVA) Commercial is Culture Sure freezing medium (Fujifilm Wako Pure Chemical Corporation). Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

Therefore, the mixture of zwitterion and DMSO is a promising freezing medium for various cell lines and even freezing-vulnerable cell. This freezing medium is very simple because they consist of only three ingredients. Commercial is further optimized by blending several additives that adjust the pH and osmotic pressure to improve the cryoprotecting effect, so it is possible to create a freezing medium that surpasses the commercial.

## 2.11 Cryoprotecting mechanisms of water/$OE_2imC_3C$/DMSO.

**Cell volume**

I investigated the cryoprotecting mechanism of freezing medium DMSO and $OE_2imC_3C$ were combined. $OE_2imC_3C$ is a non-cell-permeable cryoprotectant that strongly inhibits extracellular ice

crystal formation, and DMSO is a cell-permeable cryoprotectant that inhibits intracellular ice crystal formation.

Cell volume was measured to determine the synergistic effect of water/$OE_2imC_3C$/DMSO (Fig. 2-38). The water/DMSO mixture has a higher cell volume to the point of bursting, suggesting that DMSO requires a non-cell-permeable cryoprotectant such as $OE_2imC_3C$ or NaCl (contained in the medium). In water/$OE_2imC_3C$ (90/10, w/v), intracellular dehydration occurred because the volume of cells became smaller. The cell volume in water/$OE_2imC_3C$/DMSO (90/10/15, v/w/w) showed the same as that in water/$OE_2imC_3C$ (90/10, w/v). No significant change in cell volume was observed even when the osmotic pressure was increased. Therefore, it was suggested that intracellular dehydration occurred due to the cell-impermeable cryoprotectant, and the amount of cell-permeable cryoprotectant that entered the cells was controlled[27]. Since DMSO exists intracellularly, it is assumed that the water content of cells in water/$OE_2imC_3C$/DMSO is lower than in water/$OE_2imC_3C$. It was found that DMSO/$OE_2imC_3C$ inhibited intracellular ice crystals that had been formed by $OE_2imC_3C$ alone.

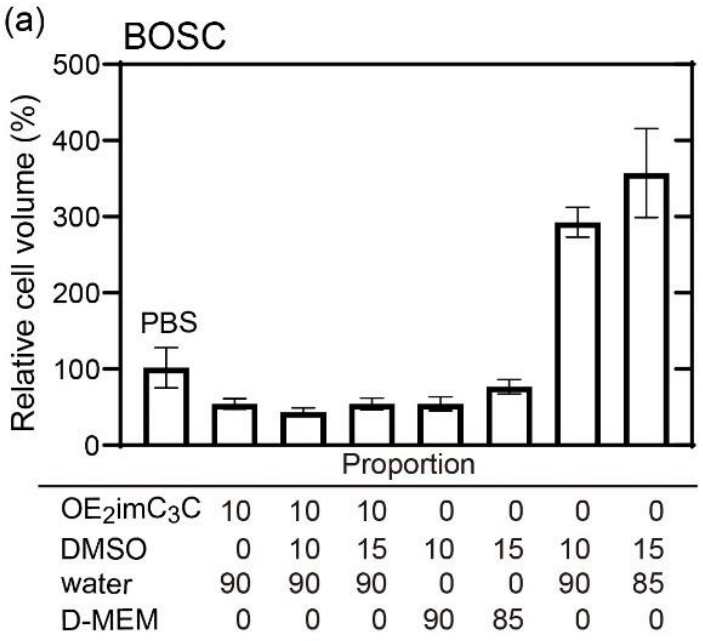



Fig. 2-38 Relative BOSC cell volumes after 5 min of immersion in the indicated solutions (cell volume in PBS was standardized as 100%). Total compositions in some cases are over 100 to be clear. The cells were immersed as floating cells in the solutions at room temperature after trypsin treatment. (n = 3, biologically triplicate, one-way ANOVA) There is no statistical significance between the samples ($p > 0.1$) Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

**Dead cell rate**

Dead cell ratio were measured to determine the synergistic effect of water/$OE_2imC_3C$/DMSO

(Fig. 2-39). The water/DMSO mixture was highly toxic and was associated with cell volume swelling as previously described. Higher concentrations of $OE_2imC_3C$ did not significantly change toxicity to cells, whereas higher concentrations of DMSO increased toxicity to cells. Interestingly, the toxicity of water/$OE_2imC_3C$/DMSO (90/10/15, v/w/w) was lower than that of D-MEM/DMSO (85/15, v/w). Therefore, $OE_2imC_3C$ may reduce the toxicity of DMSO. $OE_2imC_3C$ may inhibit DMSO influx because hyperosmotic water outflow occurs faster than DMSO influx.

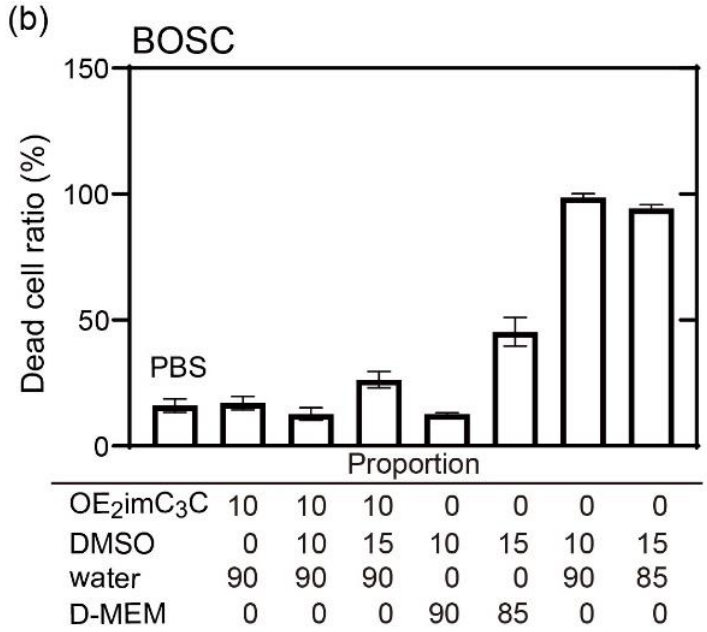



Fig. 2-39 Dead BOSC cell ratio after 60 min of immersion in the indicated solution. Total compositions in some cases are over 100 to be clear. The cells were immersed as floating cells in the solutions at room temperature after trypsin treatment. (n = 3, biologically triplicate, one-way ANOVA) There is no statistical significance between the samples ($p > 0.1$) Material from: Kato, Y. *et al.*, Synthetic zwitterions as efficient non-permeable cryoprotectants., *Communications Chemistry*, [2021], [publisher - as it appears on our copyright page]'

These results suggest that the combination of zwitterion and DMSO will be the optimal freezing medium that can be used even for freezing-vulnerable cells.

## 2.12 Conclusion

The zwitterion aqueous solutions exhibited high cryoprotecting effects through cell dehydration, inhibition of extracellular ice crystal formation, and low toxicity. By changing the structure of zwitterion, I investigated the influence of physical properties of water/zwitterion (90/10, v/w). Osmotic pressure, vitrification, glass transition temperature and the proportion of unfrozen water were not identified as key factors for cryoprotecting effect. On the other hand, cellular water content after

dehydration was an important factor for efficient cryopreservation. Although zwitterion is a promising non-cell-permeable cryoprotectant, water/zwitterion (90/10, v/w) did not show good cryoprotecting effect for all cells. Therefore, when 10% (w/v) DMSO was added as a cell-permeable cryoprotectant to compensate for the drawbacks of zwitterion, it was possible to further dehydrate the cells and reduce the toxicity of DMSO. Water/zwitterion/DMSO (90/10/15, v/w/w) was able to cryopreserve various cells. In particular, K562 cells and OMVANA cells, freezing-vulnerable cells, were cryopreserved using water/zwitterion/DMSO (90/10/15, v/w/w) more efficiently than using commercial. In summary, I found that zwitterion strongly inhibits extracellular ice crystal formation and protects cells from the negative effects of DMSO, so mixing it with DMSO provides a high cryoprotecting effect.

## 2.13 reference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## 3. Cryopreservation of cells using poly(zwitterion)

### 3.1 Introduction

In Chapter 2, DMSO was added to compensate for the shortcomings of zwitterion and to achieve high cryoprotecting effects[1]. However, rapid dehydration occurs when high osmolarities of water/zwitterion/DMSO (90/10/15, v/w/w) are applied, and the rapid dehydration has an adverse effect on cells[2, 3]. Therefore, if an isotonic freezing media can be developed, it is expected that it will be a freezing media that is gentle on cells.

Here, I focused on the fact that the salt concentration differs greatly at low temperatures. At low temperatures, most of the cryoprotectants, concentrating the salt by 10 to 20 times. Therefore, even with isotonic solutions, sufficient osmotic pressure can be obtained for dehydration at low temperatures, and slow dehydration at low temperatures is expected to cause less damage to cells than rapid dehydration. However, there is a problem in that the inside of the cell freezes because ice crystals outside of the[4] cell flow into the cell before the inside of the cell is sufficiently dehydrated[5]. In contrast, it has been reported that the amphoteric polymer (L-carboxypolylysine) developed by Matsumura *et al.* creates a matrix around the cell membrane during ice crystal formation and prevents extracellular ice crystals from flowing into cells[4, 6].

Zwitterions have a higher charge density than amphoteric polymers, and it is known that zwitterion anions strongly interact with amine cations in cell membranes[1]. Based on this, I thought that it would be possible to develop isotonic freezing media by forming a stronger matrix around cells and preventing extracellular ice crystals from flowing into cells[4, 7]. In this study, I proposed freezing media with optimal isotonicity by introducing vinyl groups into zwitterion and polymerizing it to form zwitterion polymer (polyZI) aimed to achieve highly efficient cryopreservation without using cell-permeable cryoprotectants such as DMSO.

### 3.2 Materials and Methods

**Synthesis of poly(zwitterion)**

The zwitterion monomers ($VimC_3C$, $VimC_3S$) were synthesized using a previously described method with minor modifications[8, 9].

The radical polymerization initiator (AIBN) (Tokyo Chemical Industry Co., Ltd.) was added to $VimC_3C$ in ultrapure water (Arium® Universal Kit, H2O-U-PACK, Sartorius AG.) and stirred at 80 °C for 16 h. Dialysis (Spectra/Por® 7, Pre-treated RC Tubing MWCO:1kD, Funakoshi Co., Ltd.) with ultrapure water and vacuum drying yielded poly($VimC_3C$) (Fig. 3-1). Poly($VimC_3C$) with different molecular weights were synthesized by changing the amount of AIBN relative to the zwitterion monomer. When the degree of ion substitution ($DS_{ion}$) was changed, 1-vinylimidazole (Tokyo Chemical Industry Co., Ltd.) and $VimC_3C$ were copolymerized. The structure of the synthesized poly($VimC_3C$) $DS_{ion(10–100\%)}$ was confirmed using $^1H$ and $^{13}C$ Nuclear Magnetic Resonance (NMR)

(ECA400, JEOL Ltd.) (Fig. 3-4).

The AIBN (Tokyo Chemical Industry Co., Ltd.) was added to $VimC_3S$ in 2% NaCl aqueous solution. and stirred at room temperature for 16 h. Poly($VimC_3S$) was obtained via reprecipitation in pure water and drying under reduced pressure (Fig. 3-2).

I synthesized poly($VpyC_3C$) and poly($VpyC_3S$) by slightly modifying a previously reported method[7], because a pure pyridinium-based zwitterion monomer could not be synthesized, poly(4-vinylpyridine) was zwitterionized. Poly(4-vinylpyridine) (Merck Co., Ltd.) and 1-bromo-(2-(2-methoxyethoxy))ethane (Tokyo Chemical Industry Co., Ltd.) were added to DMSO (Kanto Chemical Co., Inc.) and stirred at 80 °C for 24 h. Poly($VpyC_3C$) was obtained through washing with ethyl acetate (Kanto Chemical Co., Inc.), ion-exchange (Amberlite® IRN-78, Thermo Fisher Scientific Inc.), and drying under reduced pressure (Fig. 3-3). Poly(4-vinylpyridine) (Merck Co., Ltd.) and 1,3-propanesultone (Tokyo Chemical Industry Co., Ltd.) were added to dichloromethane (Kanto Chemical Co., Inc.) and the mixture was stirred at room temperature for 24 h and yielded poly($VpyC_3C$).

The structure of the synthesized polyZI was confirmed using $^1H$ NMR spectroscopy (ECA400, JEOL Ltd.) (Fig. 3-5). The molecular weight of the synthesized polyZI was determined using gel filtration chromatography (Prominence,Shimadzu Corporation). I used a TSKgel α-M column as the stationary phase and ultrapure water as the eluent. The flow rate was set to 1.0 mL/min, and this measurement temperature was 40 °C. The samples were analyzed using a refractive index detector (RID-10A), and the molecular weights were corrected using polyethylene oxide standards.

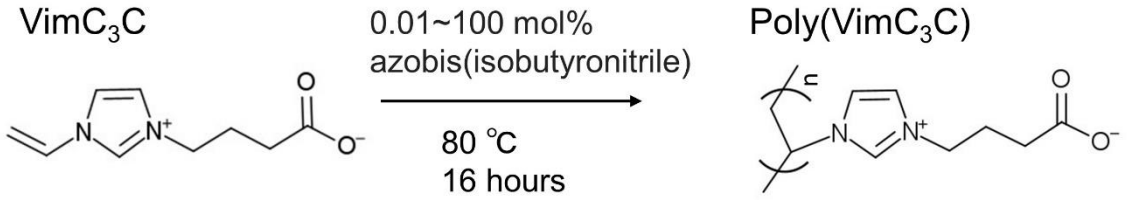


Fig. 3-1 Synthetic scheme of poly($VimC_3C$). Material from: Kato, Y. *et al.*, Optimization of zwitterionic polymers for cell cryopreservation, *Macromolecular Bioscience*, [2024], [publisher - as it appears on our copyright page]'

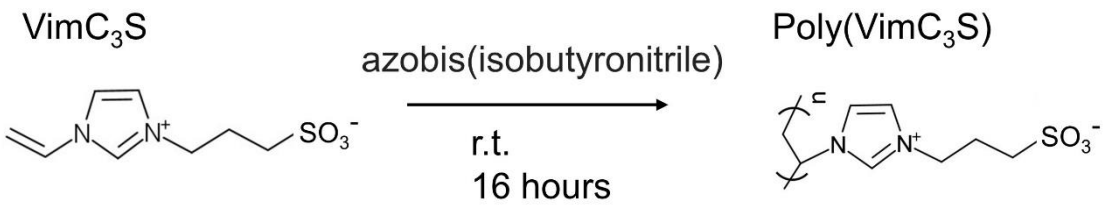


Fig. 3-2 Synthetic scheme of poly($VimC_3S$). Material from: Kato, Y. *et al.*, Optimization of zwitterionic polymers for cell cryopreservation, *Macromolecular Bioscience*, [2024], [publisher - as

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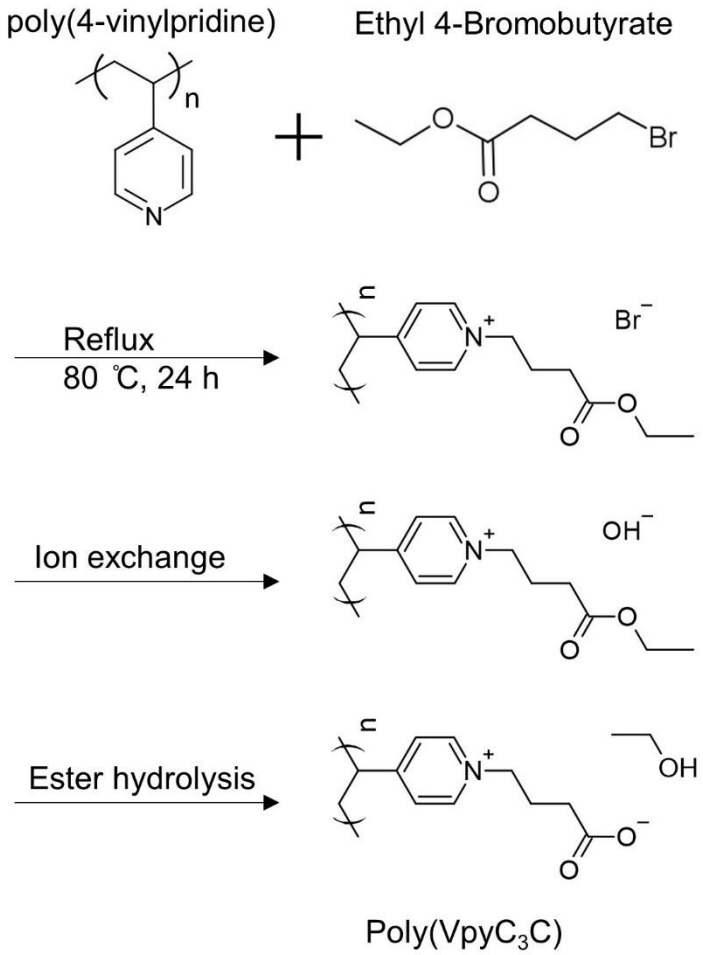
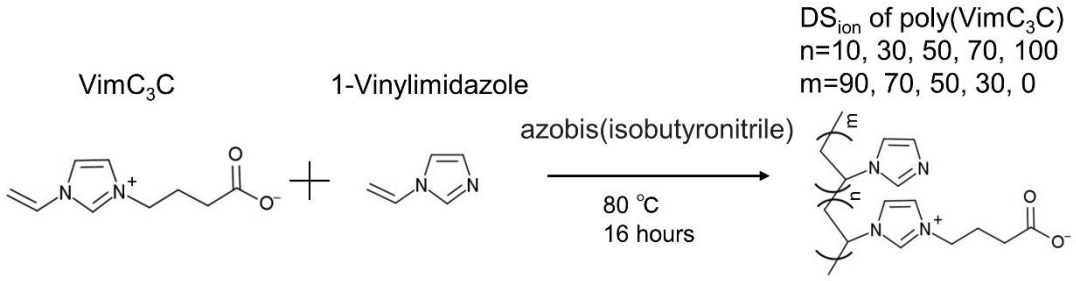



Fig. 3-3 Synthetic scheme of poly(VpyC3C). Material from: Kato, Y. *et al.*, Optimization of zwitterionic polymers for cell cryopreservation, *Macromolecular Bioscience*, [2024], [publisher - as it appears on our copyright page]'



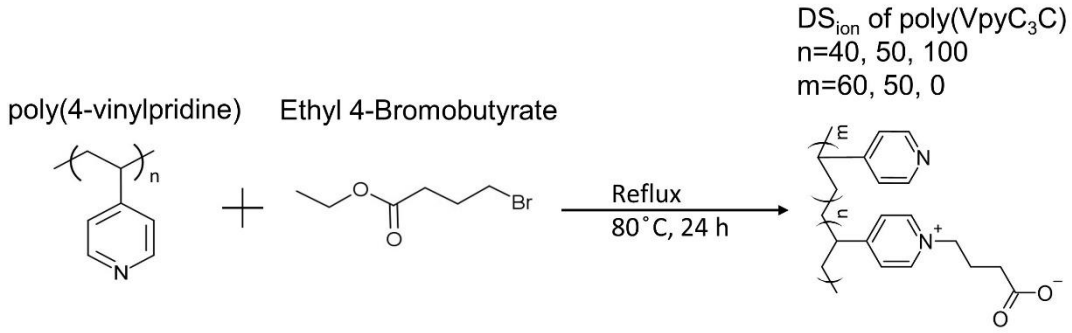



Fig. 3-4 Scheme of poly($VimC_3C$) having various $DS_{ion}$ ($DS_{ion}$: 10–100) and poly($VpyC_3C$) having various $DS_{ion}$ ($DS_{ion}$: 40–100). Material from: Kato, Y. *et al.*, Optimization of zwitterionic polymers for cell cryopreservation, *Macromolecular Bioscience*, [2024], [publisher - as it appears on our copyright page]'

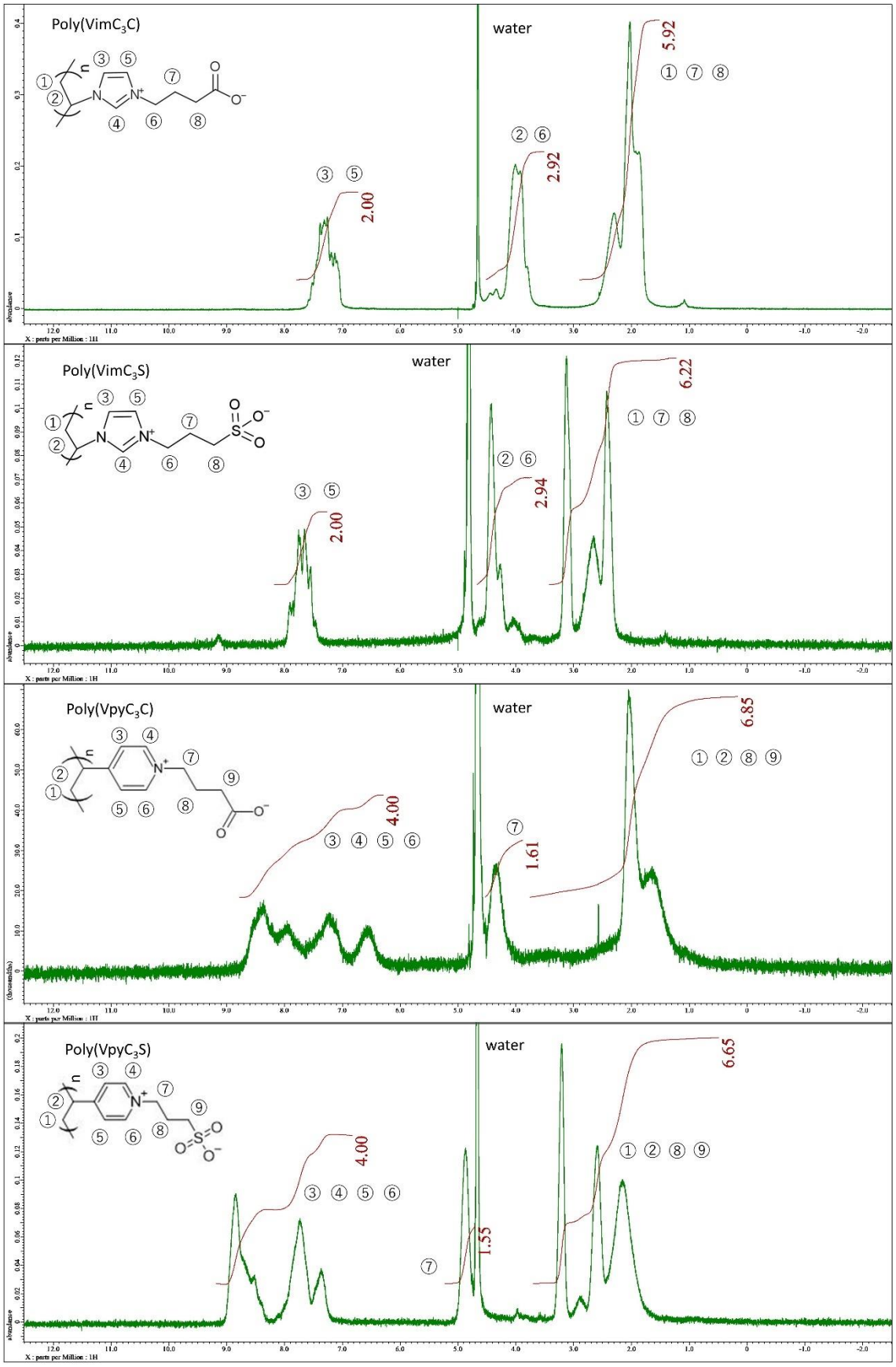

Poly(VimC3C)
water
5.92
2.92
2.00
Poly(VimC3S)
water
6.22
2.94
2.00
Poly(VpyC3C)
water
6.85
4.00
1.61
Poly(VpyC3S)
water
6.65
4.00
1.55

Fig. 3-5 NMR charts of polyZI using $D_2O$. Material from: Kato, Y. *et al.*, Optimization of zwitterionic polymers for cell cryopreservation, *Macromolecular Bioscience*, [2024], [publisher - as it appears on our copyright page]'

All reagents purchased were used as received unless stated otherwise. The reagents used to synthesize the above-mentioned polyZIs (1-vinylimidazole, 1-bromo-2-(2-methoxyethoxy)ethane, 1,3-propenesultone, azobis(isobutyronitrile), and tetrahydrofuran) were purchased from Tokyo Chemical Industry Co., Ltd., and used as received and used as is or recrystallized. The reagents used to synthesize the above-mentioned polyZIs (ethyl acetate, dimethyl sulfoxide (DMSO), and dichloromethane) were purchased from Kanto Chemical Co., Inc. The reagents used to synthesize pyridinium polyZI (poly(4-vinylpyridine)) were purchased from Merck Co., Ltd.

Trimethylglycine (TMG), DMSO and polyvinyl alcohol were purchased from Tokyo Chemical Industry Co., Ltd., and used as received. Sodium chloride, sodium polyacrylate and sucrose were purchased from Nacalai Tesque, Inc., and used as received. The ionic liquid used in the present study was purchased from Iolitech GmbH and used as received.

Ion exchange resin (Amberlite® IRN-78) was purchased from Thermo Fisher Scientific Inc. Dialysis membrane (Spectra/Por® 7, Pre-treated RC Tubing MWCO:1kD) was purchased from Funakoshi Co., Ltd. Ultrapure water unit (Arium® Universal Kit, H2O-U-PACK) was purchased from Sartorius AG.

**Cells**

I received mouse normal fibroblast (mNF) derived from C57BL/6-EGFP mice cells for kind gifts from professor Erik Sahai (The Francis-Crick Institute, UK). I received BOSC human kidney cells for kind gifts from professor Michiyuki Matsuda (Kyoto University), professor Seiji Yano (Cancer Research Institute of Kanazawa University), and professor Etsuko Kiyokawa (Kanazawa Medical University), respectively. I received K562 human chronic myelogenous leukemia cells and Vn1919 neuroglial and neuronal character co-expressing ependymoma cells used in the previous studies[10, 11]. I purchased OVMANA human ovarian tumor cells from the Japanese Collection of Research Bioresources.

**Culture**

BOSC, mNF, and Vn1919 were grown and maintained as monolayer cultures at 37 °C in 5% $CO_2$ humidified atmosphere, using Dulbecco's modified Eagle's medium (high glucose with L-glutamine and phenol red, Fujifilm Wako Pure Chemical Corporation) supplemented with 1 vol% penicillin–streptomycin solution (×100) (Fujifilm Wako Pure Chemical Corporation) and 10 vol% FBS (Sigma-Aldrich Co., Llc.). OVMANA was grown and maintained as monolayer cultures at 37 °C

in 5% $CO_2$ humidified atmosphere, using RPMI, Nacalai Tesque, Inc. supplemented with 1 vol% penicillin–streptomycin solution and 10 vol% FBS. The cells were sub-cultured every 4–6 days with trypsin solution (0.5 w/v% trypsin-5.3 mmol/L EDTA・4Na solution without phenol red (×10), Fujifilm Wako Pure Chemical Corporation).

K562 were grown and maintained as floating cultures at 37 °C in 5% $CO_2$ humidified atmosphere, using Roswell Park Memorial Institute medium (RPMI, Nacalai Tesque, Inc.) supplemented with 1 vol% penicillin–streptomycin solution and 10 vol% FBS.
These cells were sub-cultured every 4–6 days.

**Cryopreservation**

Cells ($1 \times 10^6$ cells) With or without trypsin treatment were harvested and centrifuged (100 × g, 5 min at room temperature). After removing the supernatant of samples, 100 μL of freezing media were added and pipetted gently. The samples were stored in a box (Mr. Frosty, Thermo Fisher Scientific Inc.) in a –85 °C deep freezer for 3–5 days. For the samples thawing, culture media incubated at 37 °C were added to the frozen samples. The relative number of living cells was calculated by counting using a hemocytometer (Fukaekasei Corporation and Watson Corporation) after staining using trypan blue (Fujifilm Wako Pure Chemical Corporation) and calculated.

The commercial freezing medium employed was Culture Sure freezing medium (DMSO-containing, Fujifilm Wako Pure Chemical Corporation). This is used as one of the representative commercial freezing media and is suitable for comparison in the cryoprotecting effect. In this study, the relative number of living cells was adopted because the absolute number of living cells was variable, based on biological variation, even when experiments are repeated under the same conditions and the commercial is used. The absolute numbers of living cells obtained by the commercial were approximately associated with those obtained by the sample solutions. The results still contain a certain amount of error based on the biological variation but are sufficient to suggest rough trends and relations.

$$\text{The relative number of living cells} = \frac{\text{Counted living cell number (sample)}}{\text{Counted living cell number (commercial)}}$$

**Cryopreservation solution adjustment**

Freezing media are typically prepared with concentrations based on volume/volume using pipettes; however, polyZI aqueous solution (polyZI aq.) are difficult to prepare at volume/volume due to their solid. As a result, they were prepared at volume/weight in the present study. The polyZI and NaCl solutions were prepared via mixing with ultrapure water or medium. When preparing the freezing medium, the amount of culture medium, and water were measured by volume using pipettes. The number of zwitterions, polyZI, an ionic liquid, NaCl, and sucrose were measured by weight using

an electronic balance unless I note.

**Growth rate**

All cells after cryopreservation were seeded in 6-well plates. K562 cells were counted sequentially after the indicated days. BOSC, mNF, Vn1919 and OVMANA cells were counted after trypsin treatment when most proliferating cells reached 80% confluence.

**Toxicity of cryoprotectants.**

Cells ($1 \times 10^5$ cells) after trypsin treatment were harvested and centrifuged (100 × g, 5 min at room temperature). Following the removing the supernatant of samples, 100 μL of the samples were added and pipetted gently. The cells were then incubated at room temperature (15–25 °C) as floating cells in samples. After 60 min in a floating state, the dead cell rate was calculated by counting using a hemocytometer and staining with trypan blue. Dead cell rate was defined as the following equation.

$$\text{Dead cell ratio (\%)} = \frac{\text{Number of dead cells}}{\text{Number of living cells} + \text{Number of dead cells}}$$

**Cell volumes in cryoprotectants.**

Cells ($1 \times 10^5$ cells) after trypsin treatment were harvested and centrifuged (100 × g, 5 min at room temperature). Following the removing the supernatant of samples, 100 μL of samples were added and pipetted gently. The cells were then incubated at room temperature (15–25 °C) as floating cells in samples After 5 min in a floating state, images of the cells were captured with an optical inverted microscope (IX83, Olympus Corporation). The cell radii were estimated using the "ImageJ" software (ImageJ 1.52p, Wayne Rasband, National Institutes of Health, USA). The relative cell volume was calculated using the measured cell radii, relative to the cell volume in phosphate-buffered saline (PBS). The relative cell volume was defined as the following equation.

$$\text{Relative cell volume (\%)} = \frac{\text{Cell volume in sample } (\mu m^3)}{\text{Cell volume in PBS } (\mu m^3)} \times 100$$

**Measurement of the size of polyZI aggregation**

The particle size distribution was investigated using a Dynamic Light Scattering (DLS) device manufactured by HORIBA, Ltd. The 1% (w/v) solution was passed through a 3μm filter and put into a disposable cell, and the particle size was measured at a set temperature of 25 °C and at a manual time of 120 s. After measurement, the sample was analyzed with polyvariance at a threshold of 0.

**The proportion of unfrozen water**

The proportion of unfrozen water was investigated using DSC (DSC-60A plus, Shimadzu

Corporation). DSC measurements were performed under the following conditions: cooling to −100 °C at a cooling rate of −1 °C/min followed by heating to 25 °C at a heating rate of 5 °C/min. The proportion of unfrozen water was defined as the following equation.

$$\text{Proportion of unfrozen water in solution (\%)} = 100 - \frac{\text{Melting heat of the sample solutions [J/g]}}{\text{Melting heat of water (265 [J/g])} \times \text{water proportion in the solution}} \times 100$$

**NMR**

$^{1}$H NMR was measured using ECA400 (external magnetic field 400 MHz) manufactured by JEOL Ltd. The 10% (w/v) polyZI was dissolved in $D_2O$ and measured.

**Gel Filtration Chromatography (GPC)**

The molecular weight of the polyZI was investigated using Gel Filtration Chromatography (GPC). A TSKgel α-M column was used as the stationary phase. Pure water was used as the eluent, the flow rate was 1.0 mL/min, and the measurement temperature was 40 °C. The sample was detected using a refractive index detector (RID-10A), and the molecular weight were determined by using polyethylene oxide standards.

**Ice Recrystallization Inhibition (IRI)**

A 2 μL droplet of the sample dissolved in 20% sucrose aqueous solution. was dropped onto a glass microscope coverslip. The sample was rapidly cooled from 20 °C to −50 °C at a rate of 20 ºC/min. The sample was held for 2 min at −50 °C and then warmed at 10 °C/min to −8 °C, where the sample was held for 30 min. The images of the ice crystals were photographed using a microscope with the cooling stage (ECLIPSE Ts2, Nikon Corporation). The 5-10 largest ice crystals were selected to calculate the mean largest grain size (MLGS).

**Statistical analysis**

The experimental data were subjected to one-way ANOVA followed by Dunnett test. Experimental data with two or more factors were subjected to two-way ANOVA followed by the Tukey test. These tests were performed by GraphPad Prism9.

Biological triplicate used cells harvested from the different dishes to reduce experimental errors caused by individual differences in the organisms used as experimental samples. Experimental triplicate used cells harvested from the same dish to reduce errors caused by experimental manipulation.

3.3 Cryopreservation using poly($VimC_3C$) aqueous solution

**Cell viability**

To investigate whether poly($VimC_3C$) actually exhibits a high cryoprotecting effect, I cryopreserved mNF and BOSC cells and evaluated the relative number of living cells after thawing (Fig. 3-6). When using low-molecular zwitterions, the cell viability after the cryopreservation of mNF and BOSC cells was lower than when using commercial, respectively[1]. To compare the cryoprotecting effects polymers and monomers of zwitterion, $VimC_3C$, the imidazolium/carboxylate-type zwitterion monomer, which is the starting material of poly($VimC_3C$), was used as a cryoprotectant. As expected, the relative number of living mNF and BOSC cells after cryopreservation when a 10% (w/v) $VimC_3C$ aqueous solution were low and 0.46 and 0.10, respectively. Moreover, the relative number of living mNF and BOSC cells after cryopreservation when a 10% (w/v) poly($VimC_3C$) aqueous solution were unexpectedly low and 0.02 and 0.20, respectively. This was caused by the low osmotic pressure of the poly($VimC_3C$) aqueous solution.

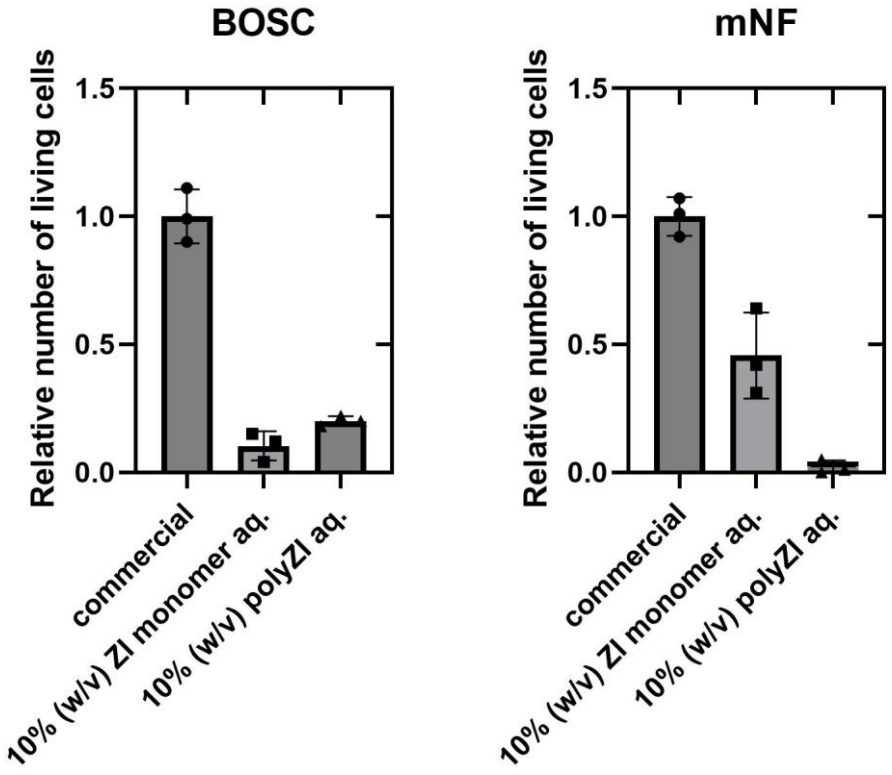



Fig. 3-6 The relative number of living mNF and BOSC cells after cryopreservation with the freezing media with poly($VimC_3C$) to compare with the cryoprotecting effect of zwitterion monomer. (n = 3, biologically triplicate, one-way ANOVA) Commercial is Culture Sure freezing medium (Fujifilm Wako Pure Chemical Corporation). Material from: Kato, Y. *et al.*, Cell-compatible isotonic freezing media enabled by thermo-responsive osmolyte-adsorption/exclusion polymer matrices,

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**Osmotic pressure**

Osmotic pressure is basically determined by the molarity of solutes. Due to the low molarity of the poly($VimC_3C$) aqueous solution, which was caused by the high-molecular-weight, it was speculated that the osmotic pressure was lower than the isotonic pressure and the cells became swollen and died. Although the osmolarity of 10% (w/v) $VimC_3C$ aqueous solution was 860 mOsm, the osmolarity of 10% (w/v) poly($VimC_3C$) aqueous solution was only 5 mOsm, which was very low (cf., culture medium: ~ 300 mOsm ; Table 3-1).

Table 3-1 The osmotic pressure and the proportion of unfrozen water in indicated solutions. The culture medium used in this study is Dulbecco's modified Eagle's medium (DMEM) with high glucose, unless noted. Material from: Kato, Y. *et al.*, Cell-compatible isotonic freezing media enabled by thermo-responsive osmolyte-adsorption/exclusion polymer matrices, *Communications Chemistry*, [2023], [publisher - as it appears on our copyright page]'

| | Osmotic pressure (mOsm) | The proportion of unfrozen water (%) |
|---|---|---|
| 10% (w/v) poly($VimC_3C$) aqueous solution | 5 | 0 |
| 10% (w/v) poly($VimC_3C$) in culture medium | 272 | 9 |
| Culture medium | 328 | 10 |
| 10% (w/v) poly($VimC_3C$) + 1.0% (w/v) NaCl aqueous solution | 289 | 4 |
| 1% (w/v) NaCl aqueous solution | 327 | 11 |
| 10% (w/v) poly($VimC_3C$) + 6.4% (w/v) sucrose aqueous solution | 157 | 8 |
| 6.4% (w/v) sucrose aqueous solution | 195 | 11 |
| 10% (w/v) poly($VimC_3C$) + 3.1% (w/v) zwitterion monomer aqueous solution | 165 | 7 |
| 3.1% (w/v) zwitterion monomer ($VimC_3C$) aqueous solution | 230 | 8 |
| 10% (w/v) poly($VimC_3C$) + 2.2% (w/v) TMG aqueous solution | 177 | 7 |
| 2.2% (w/v) TMG aqueous solution | 140 | 9 |
| 10% (w/v) poly($VimC_3C$) + 3.5% (w/v) ionic liquid aqueous solution | 253 | 14 |

| | | |
|---|---|---|
| 3.5% (w/v) ionic liquid aqueous solution | 386 | - |
| 10% (w/v) poly($VimC_3C$) in PBS | 254 | 4 |
| PBS | 280 | - |

3.4 Cryopreservation adding NaCl to the poly($VimC_3C$) aqueous solution

Osmotic pressure of 10% (w/v) poly($VimC_3C$) aqueous solution was adjusted by adding NaCl. As expected, the addition of 0.1% to 2.0% (w/v) NaCl improved the cell viability of mNF and BOSC (Fig. 3-7). In particular, the highest relative number of living mNF and BOSC cells was 0.76 and 0.84 when adding 1.0% (w/v) NaCl, which is approximately isotonic with saline and the medium (cf., 10% (w/v) poly($VimC_3C$) / 1.0% (w/v) NaCl aqueous solution: 289 mOsm; phosphate-buffered saline (PBS): 280 mOsm; culture medium: 328 mOsm). Thus, I discovered that the optimal condition for a freezing medium with polyZI would be isotonic osmolarity.

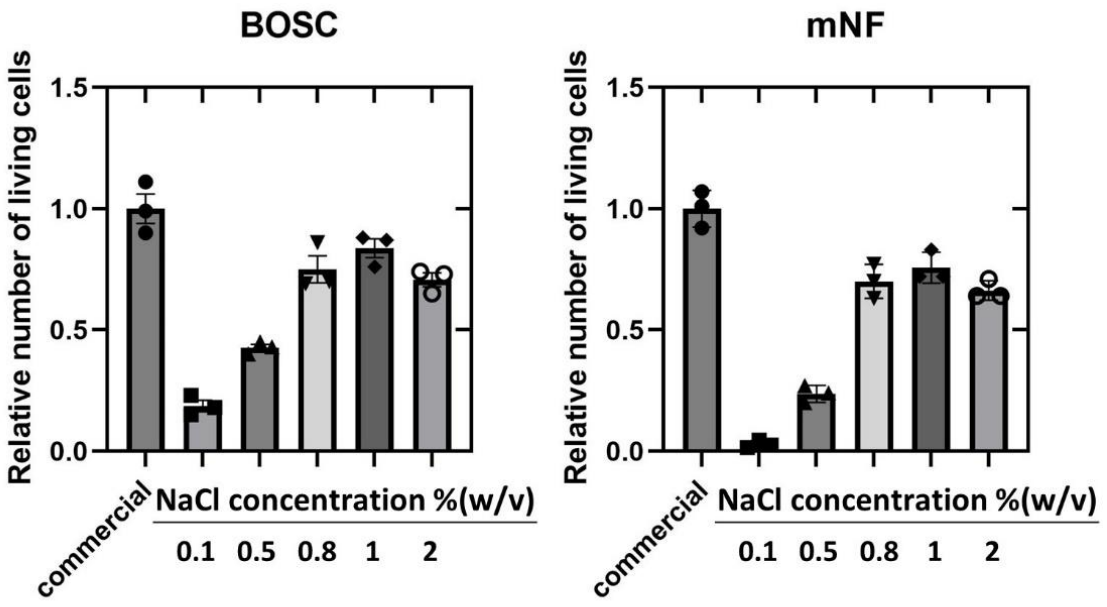


Fig. 3-7 The relative number of living mNF and BOSC cells after cryopreservation with the freezing media with poly($VimC_3C$) to compare the cryoprotecting effect of NaCl concentration. (n = 3, biologically triplicate, one-way ANOVA) Commercial is Culture Sure freezing medium (Fujifilm Wako Pure Chemical Corporation). Material from: Kato, Y. *et al.*, Cell-compatible isotonic freezing media enabled by thermo-responsive osmolyte-adsorption/exclusion polymer matrices, *Communications Chemistry*, [2023], [publisher - as it appears on our copyright page]'

It has been reported that the polyZI, especially a carboxylate-type polyZI, exhibits poor cryoprotecting performance[12]. In the present study, I could not conclude that the difference between

the previously reported polyZI and the polyZI used in this study was significant. Nevertheless, it has been shown that the polyZI structure is a necessary condition of good cryoprotectants, but not a sufficient condition.

3.5 Toxicity of poly($VimC_3C$)/NaCl aqueous solution

To confirm that the isotonic 10% (w/v) poly($VimC_3C$) / 1.0% (w/v) NaCl aqueous solution is not toxic to cells, the dead cell ratio was measured after a 60 min immersion in the freezing media at room temperature (Fig. 3-8). The dead cell ratio in the 10% (w/v) poly($VimC_3C$) / 1.0% (w/v) NaCl aqueous solution was comparable to that in the culture media. The dead cell ratio in the 1.0% (w/v) poly($VimC_3C$) / 2.0% (w/v) NaCl aqueous solution was lower than that in 2.0% (w/v) NaCl aqueous solution, indicating that osmotic shock was assumed to be alleviated, as carboxylated ε-poly-L-lysine has demonstrated previously[4].

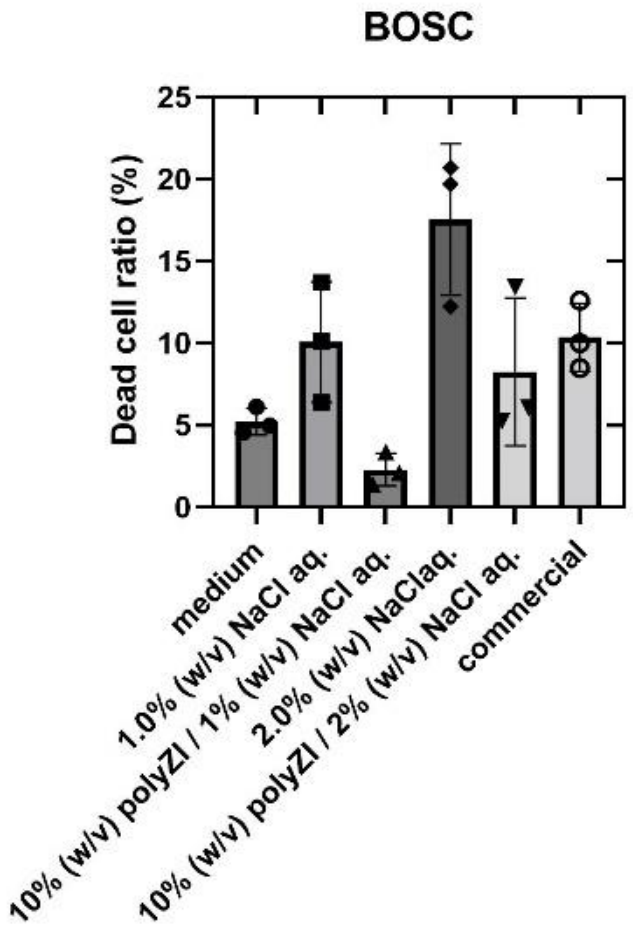


Fig. 3-8 The dead cell ratio after 60 min of immersion in isotonic poly($VimC_3C$) solution at room temperature. These cells were immersed as floating cells in 10% (w/v) poly($VimC_3C$) / 1.0% (w/v) NaCl aqueous solution after trypsin treatment. (n = 3, biologically triplicate, one-way ANOVA)

Material from: Kato, Y. *et al.*, Cell-compatible isotonic freezing media enabled by thermo-responsive osmolyte-adsorption/exclusion polymer matrices, *Communications Chemistry*, [2023], [publisher - as it appears on our copyright page]'

### 3.6 Behaviours of the poly($VimC_3C$) in NaCl aqueous solutions

**Comparison with other polymers**

An understanding of the behaviour of polyZI in freezing media is important for the clarification of the cryoprotecting mechanism of polyZI. As mentioned previously, 10% (w/v) poly($VimC_3C$) aqueous solution without NaCl had a very low osmotic pressure of 5 mOsm. Here, I compared it with other polymers. The osmotic pressures of the 10% (w/v) sodium polyacrylate (anionic polymer) and polyvinyl alcohol (electrically neutral polymer) aqueous solutions were 59 mOsm and 76 mOsm, respectively (Table 3-2). The molecular weight of poly($VimC_3C$), sodium polyacrylate, and polyvinyl alcohol were 86.3, 221.4, and 69.7 kg/mol, respectively, confirming that the extremely low osmotic pressure of poly($VimC_3C$) is not due to the molar concentration. The osmotic pressure evaluated in this study was based on the depression of vapor pressure. Considering these facts, the low osmotic pressure of polyZI aqueous solution translates to the high remaining water activity, suggesting that the polyZI does not interact strongly with water molecules. In other words, the polyZI aggregates through intramolecular charge-mediated interactions. This suggestion is supported by the 0% unfrozen water of 10% (w/v) poly($VimC_3C$) aqueous solution detected using DSC.

Table 3-2 The osmotic pressure of indicated solutions and the relative number of living mNF and BOSC cells after cryopreservation using the indicated solutions. Material from: Kato, Y. *et al.*, Cell-compatible isotonic freezing media enabled by thermo-responsive osmolyte-adsorption/exclusion polymer matrices, *Communications Chemistry*, [2023], [publisher - as it appears on our copyright page]'

| | Osmotic pressure (mOsm) | Relative number of living cells (BOSC) | Relative number of living cells (mNF) |
|---|---|---|---|
| 1% (w/v) NaCl aqueous solution | 327 | - | - |
| 10% (w/v) poly($VimC_3C$) aqueous solution | 5 | 0.07 | 0.02 |
| 10% (w/v) poly($VimC_3C$) / 1.0% (w/v) NaCl aqueous solution | 289 | 0.84 | 0.76 |
| 10% (w/v) polyvinyl alcohol aqueous solution | 76 | 0.009 | 0.03 |

| | | | |
|---|---|---|---|
| 10% (w/v) polyvinyl alcohol / 1.0% (w/v) NaCl aqueous solution | 397 | 0.02 | 0.03 |
| 10% (w/v) sodium polyacrylate aqueous solution | 59 | 0.02 | 0.03 |
| 10% (w/v) sodium polyacrylate / 1% (w/v) NaCl aqueous solution | 354 | 0.07 | 0.08 |

**Dynamic light scattering (DLS)**

In fact, I used DLS to confirm whether polyZI aggregated in water. Poly($VimC_3C$) aggregated at the molecular level in water, and aggregates with lengths of approximately 10 nm and 100 nm were observed (Fig. 3-9). The aggregated polyZI is unlikely to exhibit cryoprotecting effects; therefore, the addition of NaCl is assumed to loosen aggregation based on the electrostatic interaction. The addition of 1.0% (w/v) NaCl reduced the minimum size of poly($VimC_3C$) from 10 nm to 3 nm, confirming that aggregation was loosened (Fig. 3-10). On the other hand, the maximum size of poly($VimC_3C$) was also increased from 100 nm to 120 nm. This may have been caused by the pre-treatment of the sample; the samples were passed through filters with a pore size of 3 μm, and aggregates larger than 120 nm included in the sample without NaCl were most likely removed. However, this requires more detailed consideration.

I investigated whether the loosening effect was caused specifically by NaCl. Different types of additives were added to the poly($VimC_3C$) aqueous solution to achieve the same osmolarity as 1.0% (w/v) NaCl; sucrose, an electrically neutral sugar, and a zwitterion monomer ($VimC_3C$) and trimethylglycine (TMG), which are zwitterions. Although no clear agglomeration mitigation results were obtained, at least these additives did not loosen agglomeration. I then hypothesized that free ions may play a role in the loosening effect; therefore, a large organic ion (an ionic liquid, 1-ethyl-3-methylimidazolium acetate) was used as an additive. However, no significant change in the aggregation state was obtained; except for a slight decrease in the minimum size of the small aggregates observed at approximately 10 nm. These results indicate that free small ions, specifically NaCl, are important for the loosening of polyZI aggregation.

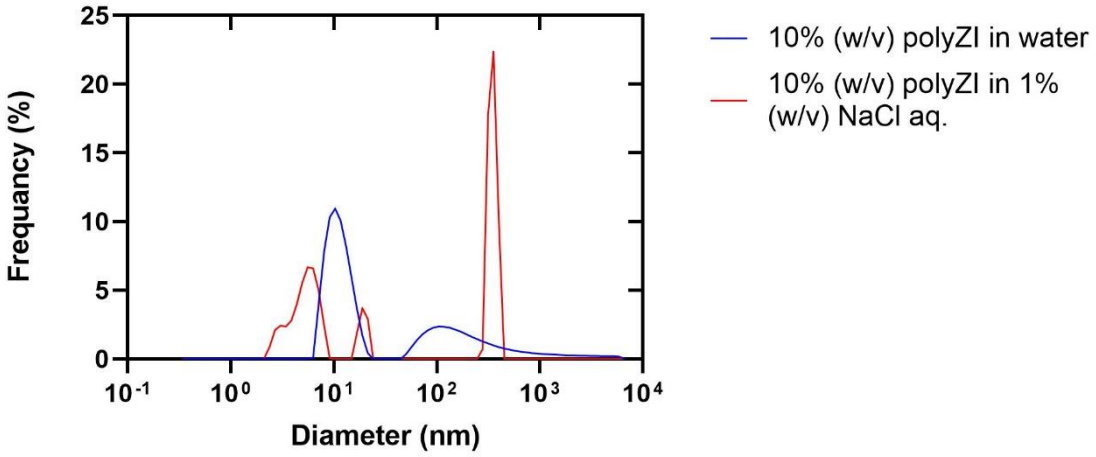


Fig. 3-9 The relationship between the size of poly($VimC_3C$) molecules and its frequency in the indicated solutions. Material from: Kato, Y. *et al.*, Cell-compatible isotonic freezing media enabled by thermo-responsive osmolyte-adsorption/exclusion polymer matrices, *Communications Chemistry*, [2023], [publisher - as it appears on our copyright page]'

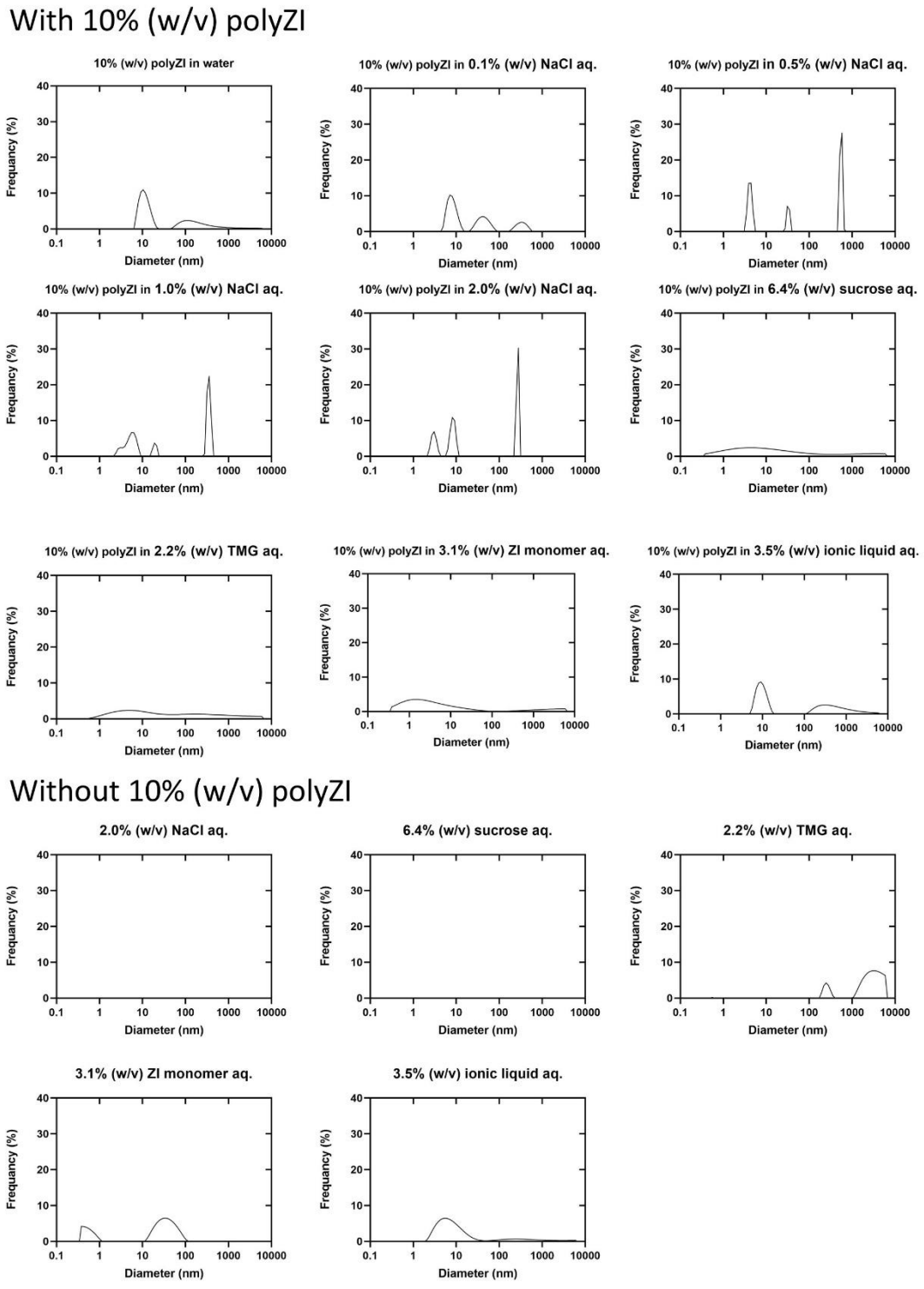


Fig. 3-10 Poly(Vim$C_3$C) sizes observed via DLS in the solutions with and without poly poly(Vim$C_3$C). The results of DLS were variable and the representative charts are shown. Material from: Kato, Y. *et al.*, Cell-compatible isotonic freezing media enabled by thermo-responsive osmolyte-adsorption/exclusion polymer matrices, *Communications Chemistry*, [2023], [publisher - as it appears

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**Nuclear magnetic resonance (NMR) spectroscopy**

The result that addition of NaCl alleviates poly(Vim$C_3$C) aggregation is consistent with the results obtained by $^1$HNMR spectra. As the NaCl concentration increased, the poly(Vim$C_3$C)-derived peaks became sharper. Thus, the addition of NaCl is assumed to loosen the aggregation and increase the molecular motion (Fig. 3-11). This loosening effect depends on the NaCl concentration.

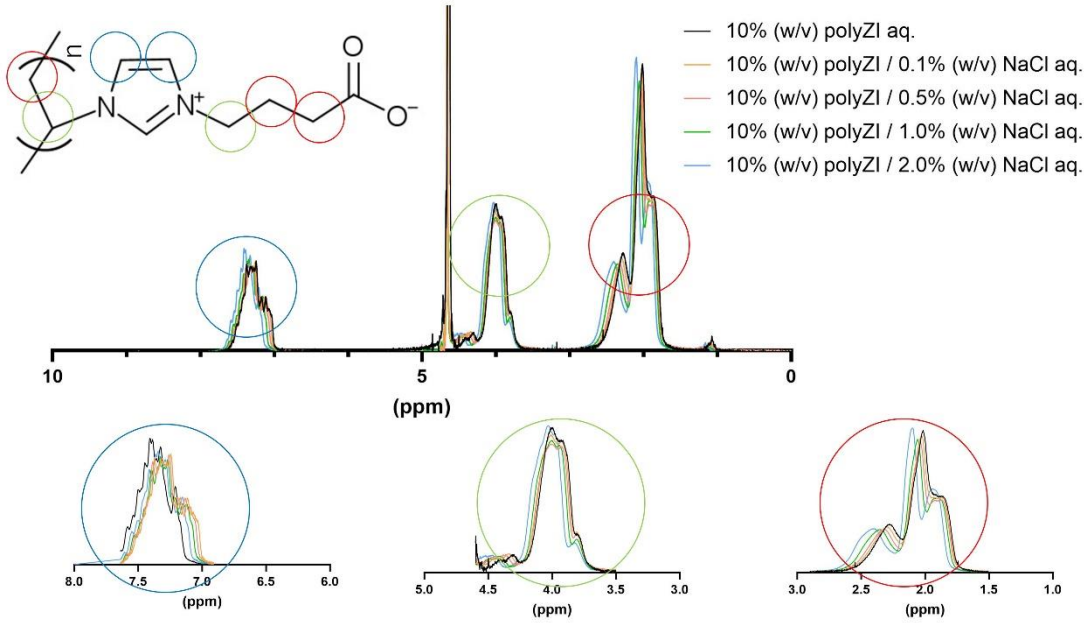


Fig. 3-11 $^1$HNM spectra of the poly(Vim$C_3$C) with 0−2% (w/v) NaCl. The solutions were prepared using deuterium oxide ($D_2O$). Material from: Kato, Y. *et al.*, Cell-compatible isotonic freezing media enabled by thermo-responsive osmolyte-adsorption/exclusion polymer matrices, *Communications Chemistry*, [2023], [publisher - as it appears on our copyright page]'

**The proportion of unfrozen water**

As mentioned above, the aggregated poly(Vim$C_3$C) scarcely interacted with water (osmotic pressure: 5 mOsm, proportion of unfrozen water: 0%) when not mixed with NaCl in 10 (w/v) % poly(Vim$C_3$C) aqueous solution. The interaction between polyZI and water and NaCl in NaCl aqueous solution was investigated. The addition of 10% (w/v) poly(Vim$C_3$C) to 1.0% (w/v) NaCl aqueous solution decreased the osmotic pressure from 327 mOsm to 289 mOsm, and the proportion of unfrozen water in the solution decreased from 11% to 4% (Table 3-1). These results suggest an increase in the water activity of polyZI and a decrease in water activity of NaCl and indicate that the NaCl interacting with water was stripped off by the polyZI; suggesting that the polyZI/NaCl interaction is stronger than that of water/NaCl. These results suggest that NaCl was adsorbed onto the polyZI, thereby changing

its structure. This strong interaction with NaCl was unique to polyZI and was not observed in the other polymers (Table 3-2).

### 3.7 Optimal additives of polyZI aqueous solution

I further investigated whether the shift of the aggregation state and interaction of polyZI plays an essential role in the cryoprotecting effect. Cells were cryopreserved using 10% (w/v) poly($VimC_3C$) aqueous solution containing the other additives at the same osmolality as 1.0% (w/v) NaCl. The relative number of living cells after cryopreservation was low when sucrose (neutral solute) and the zwitterion monomer ($VimC_3C$) and TMG (zwitterionic solutes) were used (Fig. 3-12). The relative number of living cells after cryopreservation was high when NaCl, medium (D-MEM) and PBS (free ionic solute: NaCl) were used. When an ionic liquid (organic and free ionic solute) was used, the relative number of living BOSC cells after cryopreservation was moderate, whereas that of the mNF was low. These findings are in agreement with the above-mentioned state of loosened aggregation. The aggregation loosening by adding NaCl plays an essential role in the cryoprotecting effect, based not only on an osmotic pressure adjustment, but also a structure/interaction shift.

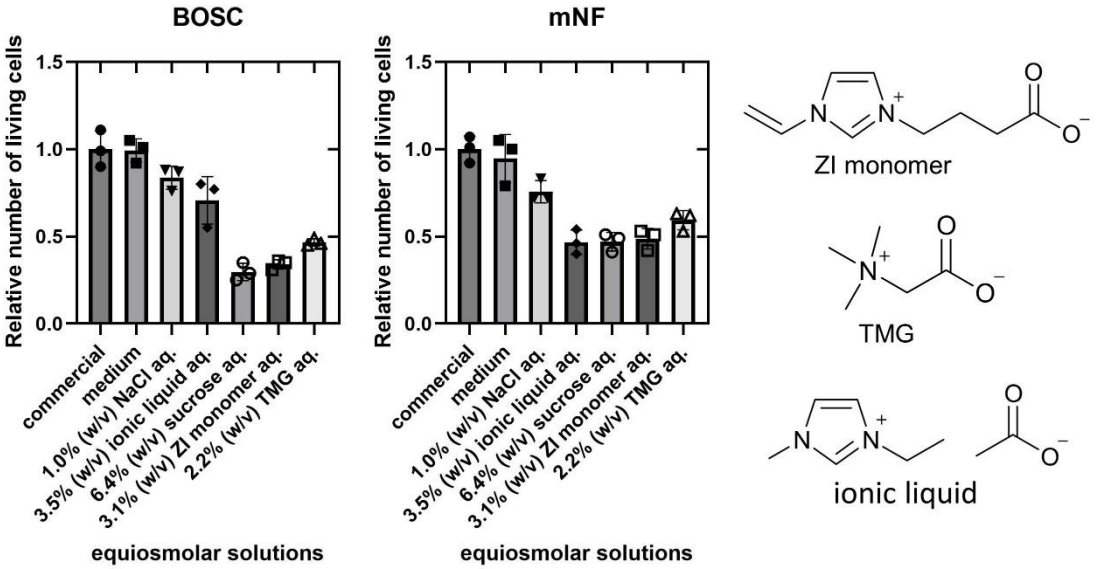


Fig. 3-12 The relative number of living mNF and BOSC cells after cryopreservation with the freezing media with poly($VimC_3C$) to compare the cryoprotecting effect of additives on poly($VimC_3C$) aqueous solutions. (n = 3, biologically triplicate, one-way ANOVA) Commercial is Culture Sure freezing medium (Fujifilm Wako Pure Chemical Corporation). Material from: Kato, Y. *et al.*, Cell-compatible isotonic freezing media enabled by thermo-responsive osmolyte-adsorption/exclusion polymer matrices, *Communications Chemistry*, [2023], [publisher - as it appears on our copyright page]'

3.8 Inhibition ice crystal formation of polyZI

**Inhibition intracellular ice crystal formation**

The primary cryoprotecting mechanism of common freezing media is associated with the inhibition of intracellular and extracellular ice crystal formation. However, the polyZI/NaCl aqueous solution does not inhibit intracellular ice crystal formation, because it is isotonic and intracellular dehydration does not occur. The relative cell volume in the 10% (w/v) poly($VimC_3C$) / 1.0% (w/v) NaCl aqueous solution and it was equivalent to that in PBS (Fig. 3-13). Therefore, the inhibition of intercellular ice crystal formation was not the primary cryoprotecting mechanism of polyZI.

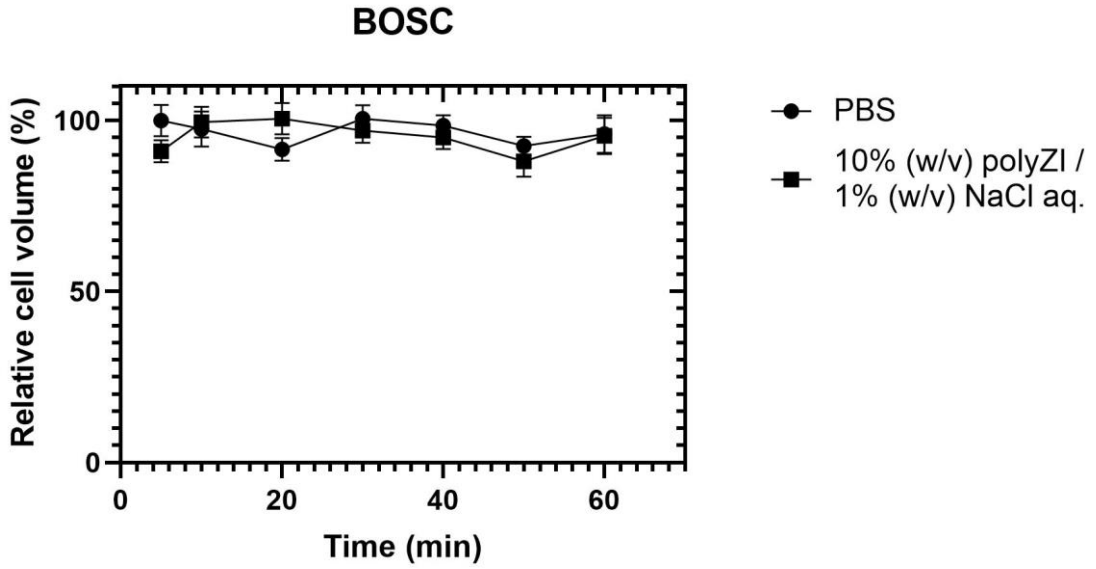


Fig. 3-13 The relative cell volume in 10% (w/v) poly($VimC_3C$) / 10% (w/v) NaCl aqueous solution and PBS (standardized as 100%) at room temperature. These cells were incubated as floating cells in the solutions after trypsinisation. (n = 3, biologically triplicate, one-way ANOVA) Material from: Kato, Y. *et al.*, Cell-compatible isotonic freezing media enabled by thermo-responsive osmolyte-adsorption/exclusion polymer matrices, *Communications Chemistry*, [2023], [publisher - as it appears on our copyright page]'

**Inhibition extracellular ice crystal formation**

Next, I investigated the inhibition of extracellular ice crystal formation of polyZI/NaCl aqueous solution. As a result of measuring the proportion of unfrozen water using DSC, the proportion of unfrozen water in 1.0% (w/v) NaCl aqueous solution was 11%, whereas the proportion of unfrozen water in 10% (w/v) poly($VimC_3C$) / 1.0% (w/v) NaCl aqueous solution decreased to 4% (Table 3-1). Furthermore, as a result of measuring the osmotic pressure of these solutions, the osmotic pressure of 1.0% (w/v) NaCl aqueous solution was 327 mOsm, whereas the osmotic pressure decreased to 289 mOsm by adding 10% (w/v) poly($VimC_3C$). In other words, it was suggested that polyZI aggregates

due to intramolecular interactions while adsorbing NaCl. The proportion of unfrozen water in 10% (w/v) poly($VimC_3C$) / 1.0% (w/v) NaCl aqueous solution was lower than those of other low-molecular zwitterion aqueous solutions, DMSO solutions, and a commercial freezing medium (10–36%)[1]. In addition, although the proportion of unfrozen water in the 10% (w/v) poly($VimC_3C$) / 6.4% (w/v) sucrose aqueous solution was 8% (Table 3-1), higher than that in 10% (w/v) poly($VimC_3C$) / 1.0% (w/v) NaCl aqueous solution, the relative number of living cells after cryopreservation was low (Fig. 3-12). This also supports the notion that the suppression of extracellular ice crystal formation is not a critical factor of cryoprotecting effect of polyZI.

**Ice recrystallisation inhibition (IRI)**

IRI activity is also an important factor of cryoprotecting effect of polyZI to consider[13, 14]. However, 10% (w/v) poly($VimC_3C$) aqueous solution with and without NaCl did not inhibit ice recrystallisation (Fig. 3-14). The ice recrystallization activity was assayed via mean largest grain size (MLGS) that is the average size of the 5-10 largest crystals from each water[9] (Fig. 3-15). There was no significant difference.

In summary, polyZI does not suppress intracellular or extracellular ice crystal formation; thus, another factor, namely the formation of polymer matrices, is assumed to be its primary cryoprotecting mechanism of polyZI.

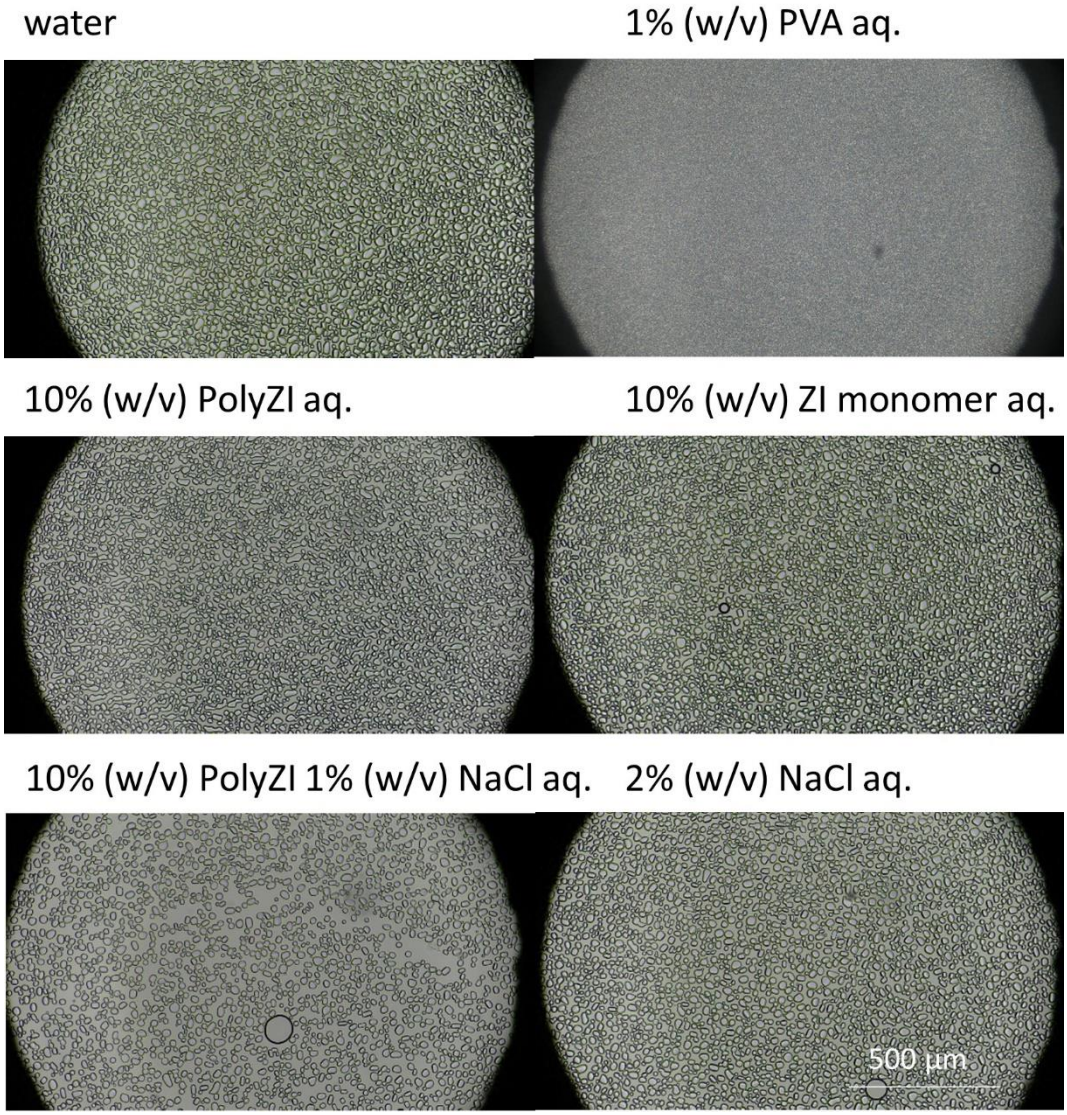


Fig. 3-14 Microscopic observation of ice formed in the indicated solutions. Material from: Kato, Y. *et al.*, Cell-compatible isotonic freezing media enabled by thermo-responsive osmolyte-adsorption/exclusion polymer matrices, *Communications Chemistry*, [2023], [publisher - as it appears on our copyright page]'

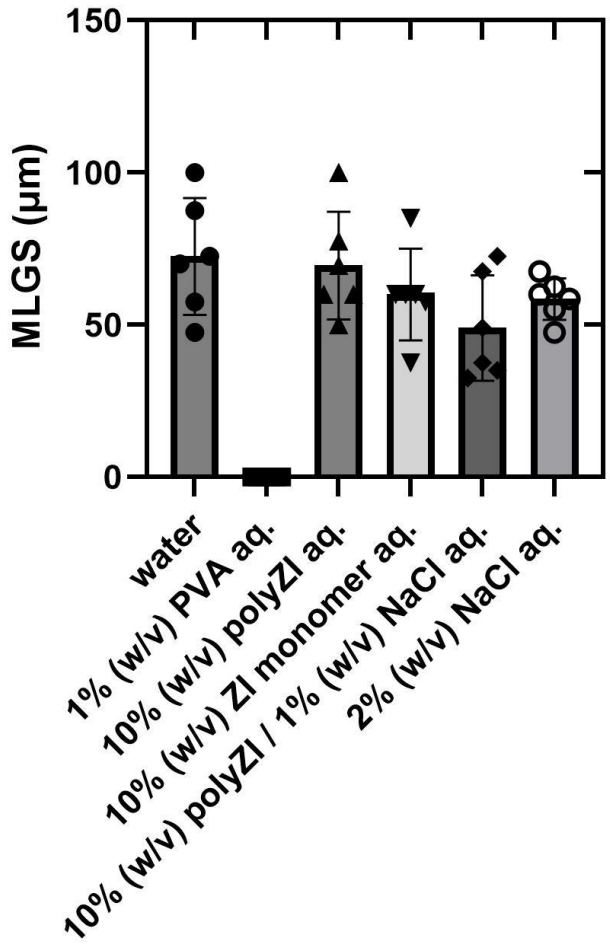


Fig. 3-15 MLGS in the indicated solutions. PVA: cannot be measured because the ice grain sizes are too small. There is no statistical significance between the samples except for 1% (w/v) PVA aq. ($p \geq 0.1$, one-way ANOVA). Material from: Kato, Y. *et al.*, Cell-compatible isotonic freezing media enabled by thermo-responsive osmolyte-adsorption/exclusion polymer matrices, *Communications Chemistry*, [2023], [publisher - as it appears on our copyright page]'

3.9 Functions of poly($VimC_3C$) matrix

I investigated the effect of the matrix formed by polyZI on suppressing the influx of extracellular ice crystals into cells. In the present study, I sought to visualize the extracellular polyZI matrix. Initially, I attempted to synthesize poly($VimC_3C$) with a fluorescent chromophore; however, the synthesis could not be achieved.

Then I observed the cell behaviour in detail and found that although the cells in PBS were evenly dispersed, the cells in 10% (w/v) poly($VimC_3C$) / 1.0% (w/v) NaCl aqueous solution adhered to each

other and formed aggregates (Fig. 3-16). This finding indirectly supports the assumption that polyZI forms matrices around the cells. It has been reported that a low-molecular zwitterion strongly interacts with cell membranes through an electrostatic interaction[15]. Since the polyZI does not interact with water, the polyZI was expanded by the addition of NaCl and then strongly interacts with the cell membrane[16, 17]and forms matrices. Therefore, the critical cryoprotecting mechanism of polyZI/NaCl aqueous solution is considered to be the polyZI matrices, inhibiting influx of extracellular ice nuclei.

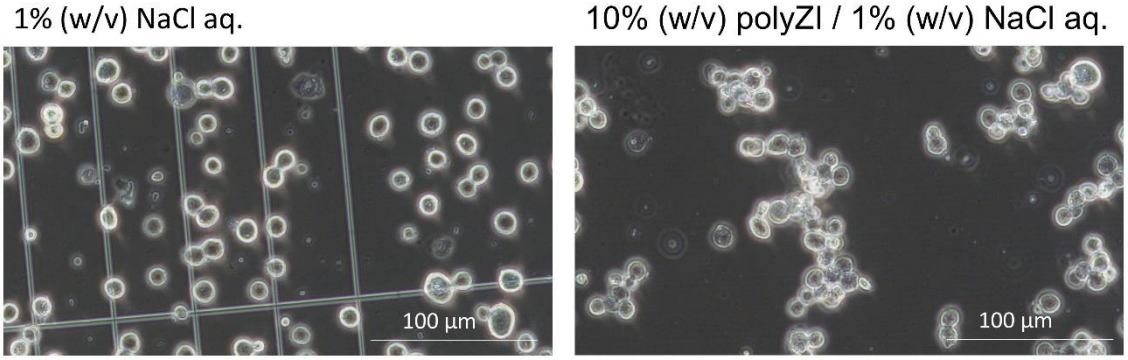


Fig. 3-16 Behaviour of BOSC cells in 1.0% (w/v) NaCl aqueous solution and 10% (w/v) poly($VimC_3C$) / 1.0% (w/v) NaCl aqueous solution BOSC cells immersed in the indicated solutions for 30 minutes were photographed using a microscope (ECLIPSE Ts2, Nikon Corporation). Material from: Kato, Y. *et al.*, Cell-compatible isotonic freezing media enabled by thermo-responsive osmolyte-adsorption/exclusion polymer matrices, *Communications Chemistry*, [2023], [publisher - as it appears on our copyright page]'

Matsumura *et al.* speculated that the matrices of carboxylated ε-poly-L-lysine, which is a polyampholyte, trap NaCl, thereby mitigating osmotic shock[4]. As shown in figure 3-8, the addition poly($VimC_3C$) reduced the dead cell ratio in hypertonic 2.0% (w/v) NaCl aqueous solution, which suggests that the polyZI matrix has the same function of polyampholytes.

The time course of osmotic pressure was estimated from the cell size (namely the volume change through cell dehydration) in 10% (w/v) poly($VimC_3C$) / 1.0% (w/v) NaCl aqueous solution to investigate the function of the matrix in detail. I found that the cell sizes in isotonic solutions were similar, regardless of the addition of poly($VimC_3C$) (Fig. 3-13). When the temperature was lowered at -1 °C/min, the cells were gently dehydrated in 10% (w/v) poly($VimC_3C$) / 1.0% (w/v) NaCl aqueous solution but not in the NaCl aqueous solution (Fig. 3-17). Thus, dehydration was affected by temperature rather than soaking time. This reaction was unique to polyZI/NaCl, as it was not observed in NaCl aqueous solution. The temperature-dependent dehydration indicates that the polyZI matrix locally concentrated NaCl at subzero temperatures.

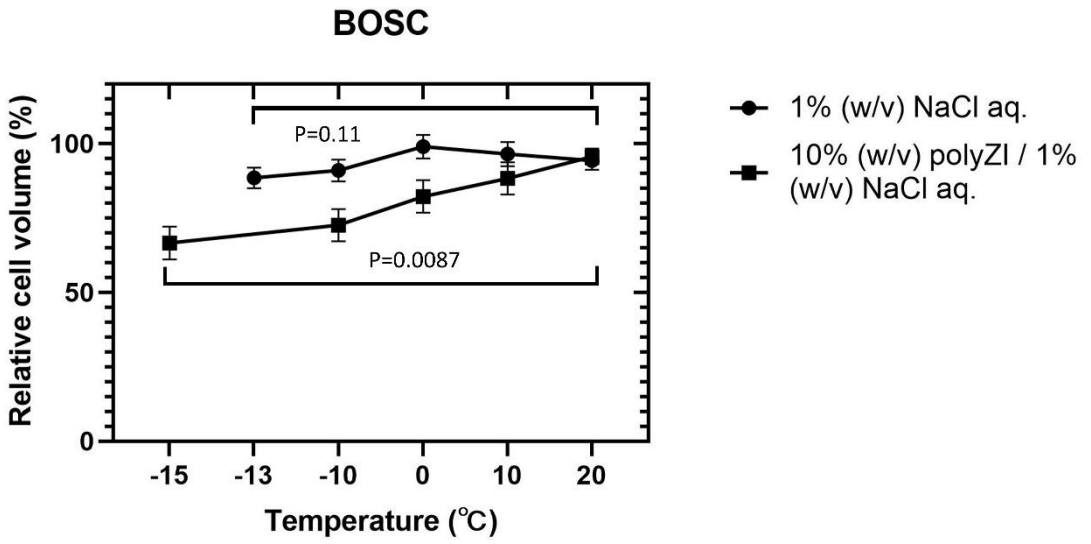


Fig. 3-17 The relative BOSC cell volume in 10% (w/v) poly($VimC_3C$) / 1.0% (w/v) NaCl aqueous solution and 1.0% (w/v) NaCl aqueous solution at the indicated temperatures (standardized as 100% at room temperature) when cooling at -1 °C/min. These cells were incubated as floating cells after trypsinization. The final cell volume was measured immediately before freezing as measurement becomes difficult after freezing. (n = 3, biological triplicates, one-way ANOVA) Material from: Kato, Y. *et al.*, Cell-compatible isotonic freezing media enabled by thermo-responsive osmolyte-adsorption/exclusion polymer matrices, *Communications Chemistry*, [2023], [publisher - as it appears on our copyright page]'

In addition to preventing influx of extracellular ice nuclei, temperature-dependent dehydration may contribute to the inhibition of intracellular ice crystal formation. Here I discuss this, referring to cryopreservation using low-molecular zwitterions. Cell dehydration is the main cryoprotecting mechanism of low-molecular zwitterions, and the relative cell volume in 10% (w/v) $VimC_3C$ aqueous solution becomes 40% or less at 0 °C compared to that in a culture medium at room temperature[1]. The relative cell volume at 0 °C and -13 °C in 10% (w/v) poly($VimC_3C$) / 1.0% (w/v) NaCl aqueous solution was 81% and 66%, respectively. This is indicated that the dehydration by polyZI/NaCl alone may not be sufficient but may help the inhibition of intracellular ice crystal formation together with the polyZI matrices. More interestingly, the volume returned to its original state when the temperature increased (Fig. 3-18), suggesting that adsorption/desorption was reversible and the osmotic pressure returned to normal during thawing. The cryoprotecting mechanism of polyZI is not only strengthening the polymer matrix formation but also the reversible adsorption/desorption, which is partially different from that reported for polyampholytes.

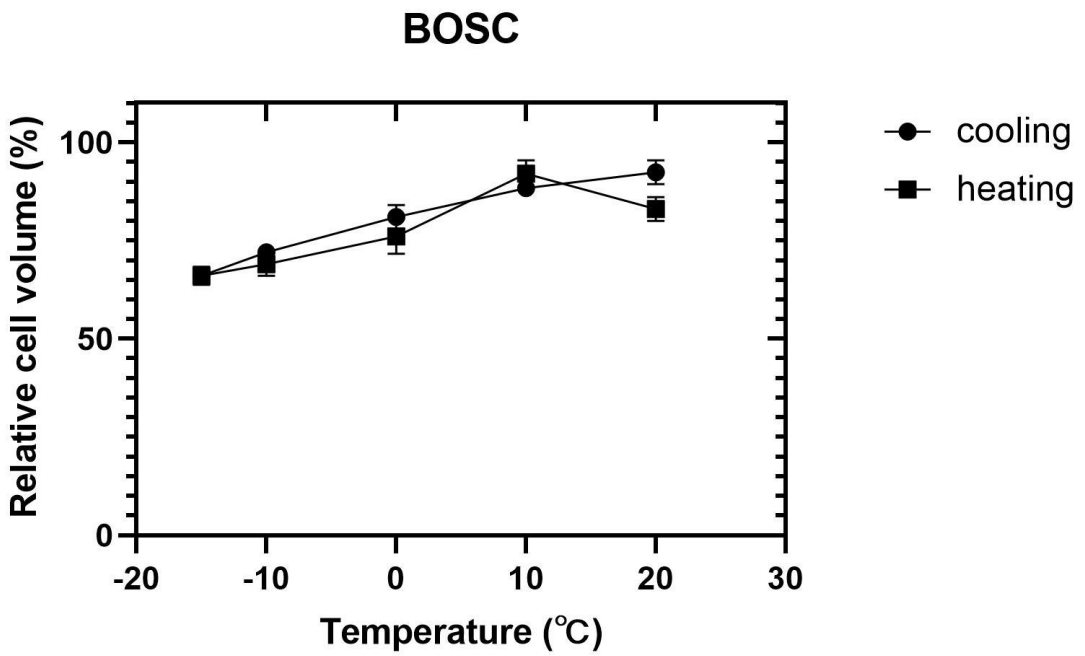


Fig. 3-18 The Relative BOSC cell volume in 10% (w/v) poly(Vim$C_3$C) / 1.0% (w/v) NaCl aqueous solution during cooling and then heating at ±1 °C/min. These cells were incubated as floating cells after trypsinisation. (n = 3, biological triplicates) Material from: Kato, Y. *et al.*, Cell-compatible isotonic freezing media enabled by thermo-responsive osmolyte-adsorption/exclusion polymer matrices, *Communications Chemistry*, [2023], [publisher - as it appears on our copyright page]'

The polyZI has triple functions and is suitable for cryopreservation: (1) firm polyZI matrices inhibit inflow of extracellular ice nuculei, (2) polyZI floating in the solution absorbs NaCl to alleviate osmotic shock at room temperature, (3) the extracellular polyZI matrices concentrate NaCl to dehydrate and suppress intracellular ice crystal formation at subzero temperatures. To date, the functions (2) and (3) have not been reported in polyampholytes. More specifically, to the best of our knowledge, the function (3) has not been previously reported, while the function (2) has only been reported at different and specific conditions: after freezing and thus not at room temperature[4]. Although, it is not clear whether these functions are specifically exhibited in polyZI only, and not all polyampholytes, the polyZI is assumed to adsorb NaCl more strongly than polyampholytes due to its charge. If the polyZI structure is a key factor, I speculate that the more specific key factor is: the polyZI is an aprotic polymer imparting constant charges, while carboxylated ε-poly-L-lysine is a protic polymer exhibiting an equilibrium of neutral/charged states. I believe that these unique functions based on the unique characteristics play a key role in the cryoprotecting mechanism under isotonic conditions.

3.10 Proliferation problem by matrix

In cryopreservation, freshly thawed cell viability can be used for initial screening; however, even high values can lead to false positives[18]. In this case, the cells survived even though they were severely damaged, and a higher cell viability than the original was obtained. To test this, I examined the number of cryopreserved cells after several days of culturing. Although the number of living cells after cryopreservation with 10% (w/v) poly($VimC_3C$) / 1.0% (w/v) NaCl aqueous solution immediately after thawing was not significantly different from that that with the commercial, the proliferation of cells cryopreserved with poly($VimC_3C$)/NaCl aqueous solution was significantly slower (Fig. 3-19). I observed the cells in detail and found that cell adhesion to the dishes was less when cryopreserved using 10% (w/v) poly($VimC_3C$) / 1.0% (w/v) NaCl aqueous solution. The polyZI matrix could inhibit of adhesion. In this experiment, the freezing medium (100 μL) was washed once by adding culture medium (1 mL) and centrifuging. These results suggest that this washing process was not appropriate for the removal of the polyZI matrix.

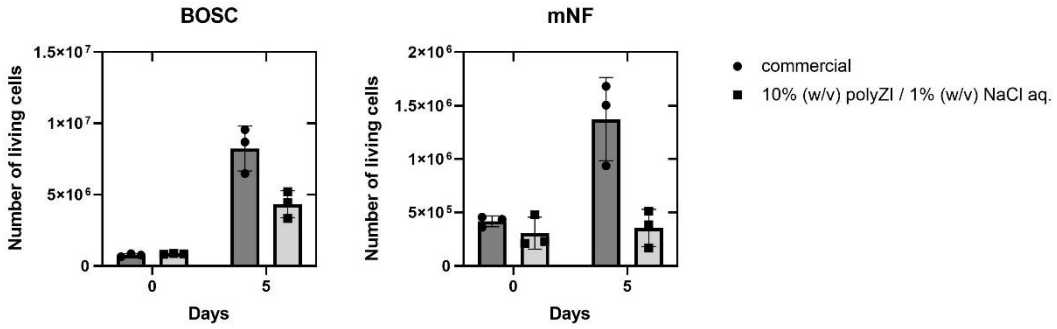


Fig. 3-19 Number of living BOSC and mNF cells immediately and 5 days after freezing and thawing in 10% (w/v) poly($VimC_3C$) / 1.0% (w/v) NaCl aqueous solution. There is no statistical significance between the samples ($p > 0.1$) Commercial is Culture Sure freezing medium (Fujifilm Wako Pure Chemical Corporation). ($n = 3$, biological triplicates, one-way ANOVA) Material from: Kato, Y. *et al.*, Cell-compatible isotonic freezing media enabled by thermo-responsive osmolyte-adsorption/exclusion polymer matrices, *Communications Chemistry*, [2023], [publisher - as it appears on our copyright page]'

3.11 Optimization of poly(zwitterion)

Therefore, it is necessary to optimize polyZI in order for cells to proliferate after cryopreservation. In this study, I investigated the molecular weight, cations, anions, degree of ion substitution ($DS_{ion}$), concentration, and washing method of polyZI. This time, I performed optimization based on poly($VimC_3C$), which was also used above.

**Molecular weights**

First, I investigated the optimal molecular weight. Poly($VimC_3C$) with different molecular weights was synthesized by varying the amount of the radical polymerization initiator (azobis(isobutyronitrile), (AIBN)), from 0.01 to 100 mol% relative to the zwitterion monomer (Fig. 3-1). The poly($VimC_3C$) used in a previous study[7]was synthesized using 1 mol% AIBN. The molecular weights of poly($VimC_3C$) were similar when 0.01 and 0.1 mol%, and 10 and 100 mol% of AIBN were added (Mw:$9.8\times10^4$ and $1.5\times10^4$, respectively). Poly($VimC_3C$) synthesized by adding 0.1, 1, 10 mol% of AIBN was used (Mw:$9.8\times10^4$, $2.8\times10^4$, and $1.5\times10^4$, respectively).

mNF and BOSC were cryopreserved using synthesized poly($VimC_3C$) with different molecular weights. The cryoprotective effect did not depend on the molecular weight (Fig. 3-20). It has been reported that IRI activity, which is thought to be related to the cryoprotective effect, is higher as the molecular weight of the polymer is larger[9, 19-21]. This is because as the molecular weight increases, the number of molecules that can bind to the ice surface increases, and by binding and pinning the ice surface, it inhibits ice growth. However, poly($VimC_3C$) does not show this relationship because poly($VimC_3C$) showed less ice-recrystallization inhibition activity than polyvinyl alcohol, as assayed by the MLGS (Fig. 3-21). The intermediate size of poly($VimC_3C$) (MW:$2.8\times10^4$) was subjected to subsequent experiments.

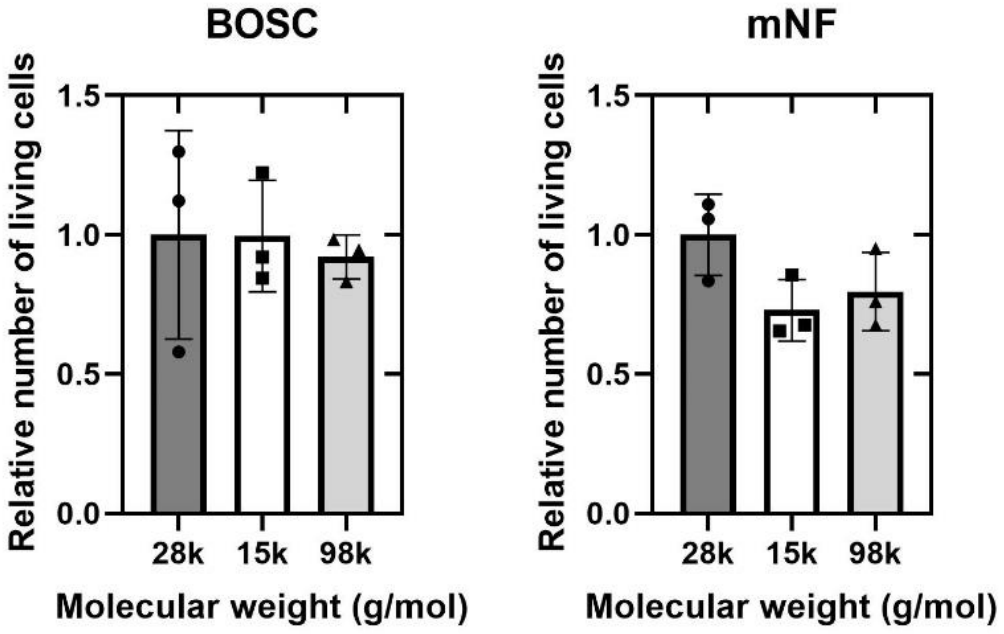


Fig.3-20 The relative numbers of living BOSC and mNF after cryopreservation using poly($VimC_3C$) with different molecular weights. Freezing medium:10% (w/v) poly($VimC_3C$) / 1% (w/v) NaCl aqueous solution. Original solution developed in the previous study is 10% (w/v) 28kg/mol poly($VimC_3C$) / 1% (w/v) NaCl aqueous solution. No statistically significant differences were

observed between the samples ($p > 0.1$). ($n = 3$, biological triplicates, one-way ANOVA) Material from: Kato, Y. *et al.*, Optimization of zwitterionic polymers for cell cryopreservation, *Macromolecular Bioscience*, [2024], [publisher - as it appears on our copyright page]'

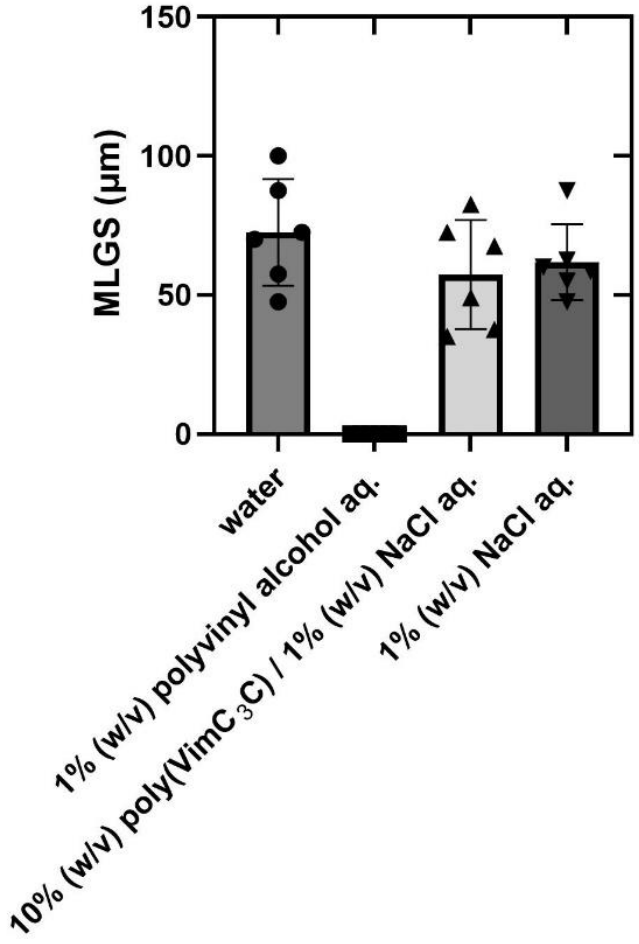


Fig. 3-21 MLGS in the indicated solutions. PVA: cannot be measured because the ice grain sizes are too small. There is no statistical significance ($p \geq 0.1$, one-way ANOVA) between the samples except for 1% (w/v) PVA aqueous solution. Material from: Kato, Y. *et al.*, Optimization of zwitterionic polymers for cell cryopreservation, *Macromolecular Bioscience*, [2024], [publisher - as it appears on our copyright page]'

**Anions**

I synthesized polyZI with carbonate-based and sulfonate-based anions and compared their cryoprotecting effects. The sulfonate-based polyZI (poly($VimC_3S$), Fig. 3-2) is a potential candidate as better non-cell-permeable cryoprotectants but is insoluble in water[7]. However, because I have also reported that the addition of NaCl loosens the aggregation of poly($VimC_3C$) in water, NaCl should

make poly(Vim$C_3$S) soluble. Studies have assessed solubilizing sulfonate-based polyZIs in NaCl aqueous solution[22, 23]. Poly(Vim$C_3$S) dissolved in 3% (w/v) NaCl aqueous solution, but not at low NaCl concentrations.

When the cells were cryopreserved using 10% (w/v) poly(Vim$C_3$S) / 3% (w/v) NaCl aqueous solution, the relative number of living cells was low (Fig. 3-22). A possible reason for this low viability is the high osmotic pressure of 3% (w/v) NaCl aqueous solution (approximately three times that of the medium). The same concentration of NaCl decreased the number of living cells, even when poly(Vim$C_3$C) was used. However, the cryoprotective effects of poly(Vim$C_3$S) were weaker than those of poly(Vim$C_3$C). Although there was no significant difference in the cryoprotective effects of carboxylate- and sulfonate-type low-molecular-weight zwitterions, carboxylate polyZIs were suitable.

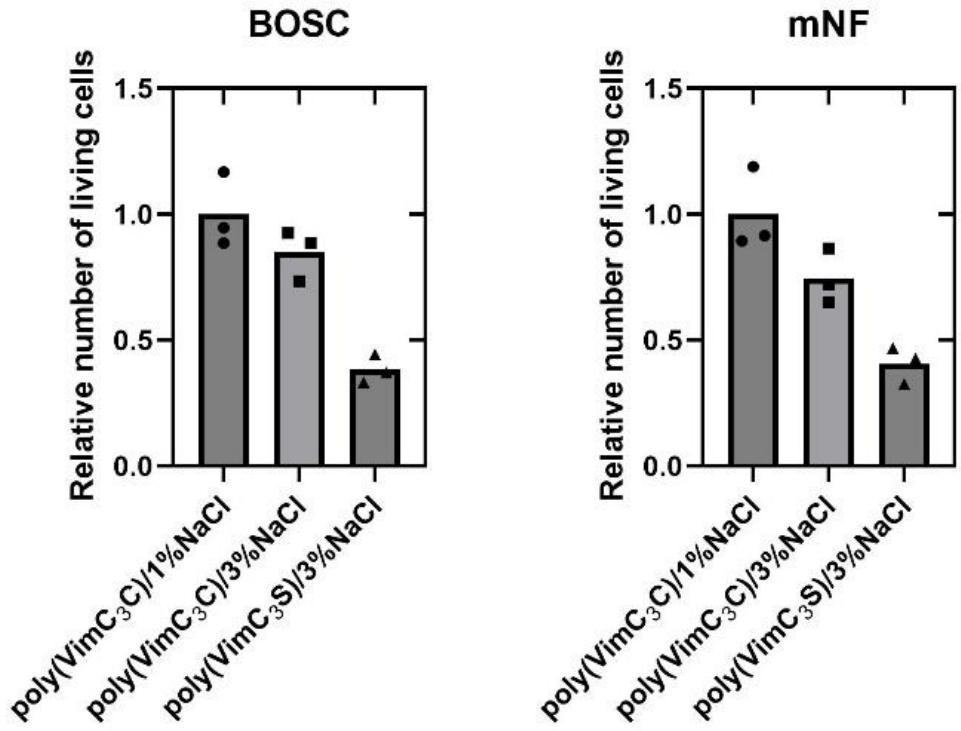


Fig. 3-22 The relative numbers of living BOSC and mNF cells after cryopreservation using polyZI with different anions. Freezing medium:10% (w/v) polyZI/NaCl aqueous solution. Original solution developed in the previous study is 10% (w/v) poly(Vim$C_3$C) / 1% (w/v) NaCl aqueous solution. (n = 3, experimental triplicates) Material from: Kato, Y. *et al.*, Optimization of zwitterionic polymers for cell cryopreservation, *Macromolecular Bioscience*, [2024], [publisher - as it appears on our copyright page]'

**Cations**

I synthesized polyZI with imidazolium-based and pyridinium-based cations and compared their

cryoprotecting effects. A pyridinium-based polyZI (poly($VpyC_3C$)) was synthesizedusing different methods (Figs. 3-3). Because a pure pyridinium-based zwitterion monomer could not be synthesized, poly(4-vinylpyridine) was zwitterionized. Poly($VpyC_3C$) was soluble in water. Pyridinium/sulfonate polyZI (poly($VpyC_3S$)) did not dissolve in low-concentration NaCl aqueous solution, similar to poly($VimC_3S$), but dissolved in 6% (w/v) NaCl aqueous solution.

Poly($VpyC_3C$) had a lower cryoprotective effect than poly($VimC_3C$) (Fig. 3-23). This trend approximately corresponds to the case of low-molecular-weight zwitterions[7]. A low number of living cells was observed when poly($VpyC_3S$) was used because the NaCl concentration was too high (Fig. 3-24).

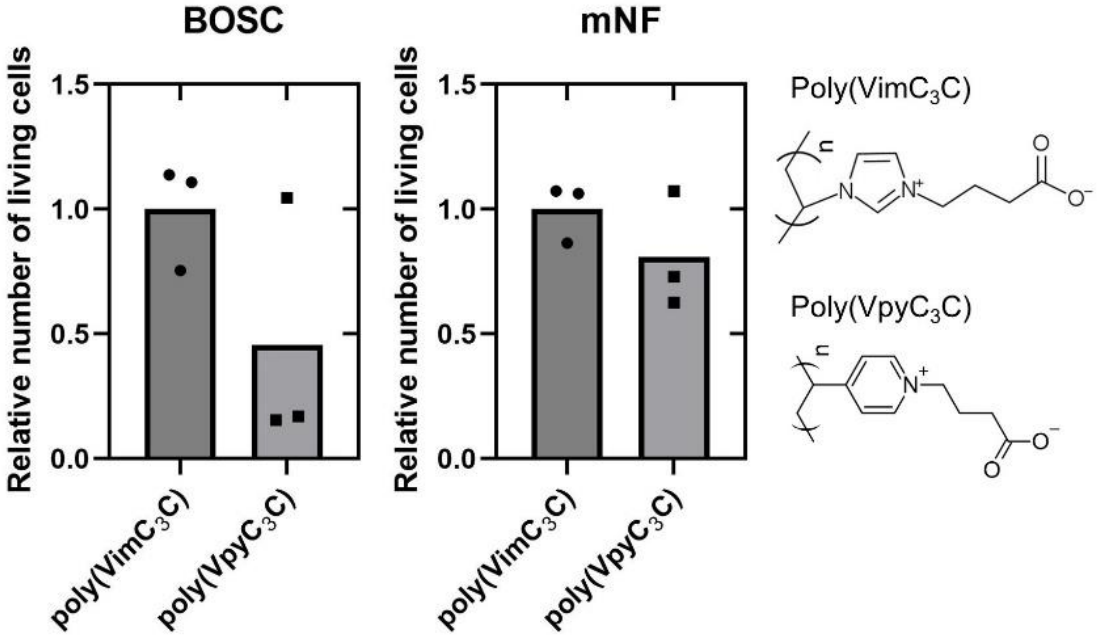


Fig. 3-23 The relative number of living BOSC and mNF cells after cryopreservation using 10% (w/v) polyZI/ 1% (w/v) NaCl aqueous solution with different cations. Original solution developed in the previous study is 10% (w/v) poly($VimC_3C$) / 1% (w/v) NaCl aqueous solution. (n = 3, experimental triplicate) Material from: Kato, Y. *et al.*, Optimization of zwitterionic polymers for cell cryopreservation, *Macromolecular Bioscience*, [2024], [publisher - as it appears on our copyright page]'

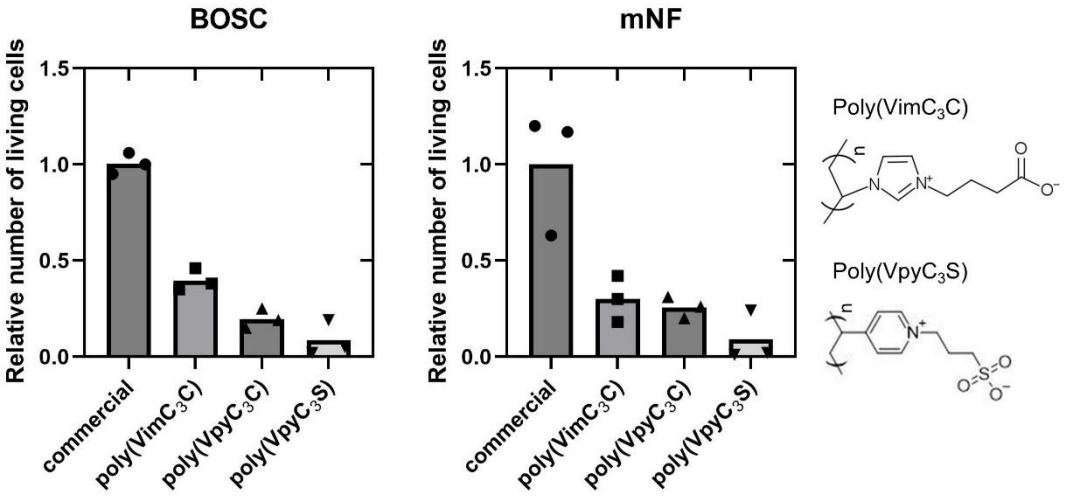


Fig. 3-24 The relative number of living BOSC and mNF cells after cryopreservation using polyZI with 6% NaCl aqueous solution. Commercial: Culture Sure freezing medium from Fujifilm Wako Pure Chemical Corporation. (n = 3, experimental triplicates) Material from: Kato, Y. *et al.*, Optimization of zwitterionic polymers for cell cryopreservation, *Macromolecular Bioscience*, [2024], [publisher - as it appears on our copyright page]'

**Degree of ion substitution ($DS_{ion}$) of polyZI**

PolyZI strongly interacts with cell membranes. Therefore, I thought that by optimizing the $DS_{ion}$ of polyZI, the interaction between polyZI and cell membranes might be adjusted, making removal after cryopreservation possible. PolyZIs with reduced $DS_{ion}$ were synthesized (Fig. 3-4); however, their affects were found to be non-significant (Fig. 3-25, Table 3-3).

Here I thought that poly($VimC_3S$) with low $DS_{ion}$ may dissolve in 1% NaCl aqueous solution. However, poly($VimC_3S$) with 10 and 40% $DS_{ion}$ did not dissolve in water. Surprisingly, they were insoluble even in a saturated NaCl aqueous solution. These results indicate that poly($VimC_3S$) is difficult to use as a cryoprotectant.

Furthermore, I thought that changing the $DS_{ion}$ of polyZI would change the interaction between polyZI in the solution, so I dissolved it in various solvents. In the case of poly($VpyC_3C$), more than 40% $DS_{ion}$ was dissolved in water. Even if the $DS_{ion}$ was changed, all $DS_{ion}$ were soluble in methanol. 20–30% $DS_{ion}$ was not dissolved unless the NaCl concentration was saturated, and 10% $DS_{ion}$ was soluble only in DMSO. Poly($VpyC_3C$) was not dissolved in acetone. In other words, Poly($VpyC_3C$)_$DS_{ion(40\%–50\%)}$ was a hydrophilic polymer. In the case of poly($VpyC_3S$), all $DS_{ion}$ did not dissolve in water, methanol, and acetone, but dissolved in DMSO. 40% $DS_{ion}$ was dissolved only in DMSO. 10% $DS_{ion}$ was dissolved only in ethanol and DMSO. In this way, changing $DS_{ion}$ showed completely different behavior.

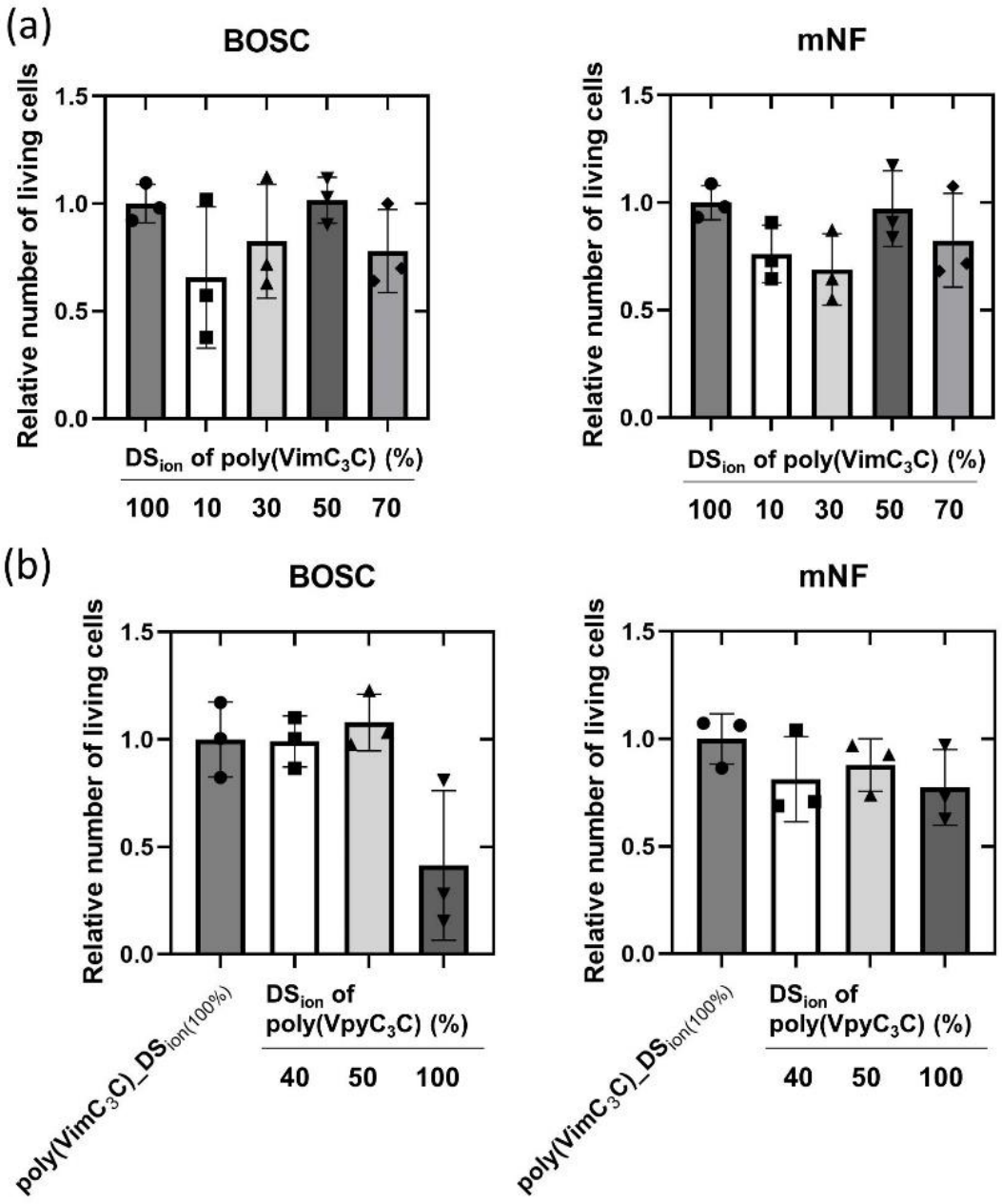


Fig. 3-25 The relative numbers of living BOSC and mNF cells after cryopreservation in (a) 10% (w/v) poly($VimC_3C$) / 1% (w/v) NaCl aqueous solution, and (b) 10% (w/v) poly($VpyC_3C$) / 1% (w/v) NaCl aqueous solution using different $DS_{ion}$. No statistically significant differences were observed between the results obtained using the polyZI sample. Freezing medium:10% (w/v) polyZI / 1% (w/v) NaCl aqueous solution. Original solution developed in the previous study is 10% (w/v) poly($VimC_3C$)_$DS_{ion(100\%)}$ / 1% (w/v) NaCl aqueous solution. No statistically significant differences

were observed between the samples ($p > 0.1$). ($n = 3$, biological triplicates, one-way ANOVA) Material from: Kato, Y. *et al.*, Optimization of zwitterionic polymers for cell cryopreservation, *Macromolecular Bioscience*, [2024], [publisher - as it appears on our copyright page]'

It is considered possible to discuss the influence of cryoprotecting effect of poly($VimC_3C$) by confirming the behavior of poly($VimC_3C$) with varying molecular weight and $DS_{ion}$. Differential scanning calorimetry of poly($VimC_3C$) solutions was performed. No significant change in Proportion of unfrozen water (bound water) was observed even when the molecular weight was changed. Proportion of unfrozen water in poly($VimC_3C$)/1% (w/v) NaCl aqueous solution increased as $DS_{ion}$ decreased. These results indicate that lowering $DS_{ion}$ relaxes the aggregates of poly($VimC_3C$) dispersed in water, allowing them to interact with water. The relaxation of the aggregate structure facilitated the formation of the polymer matrix around the cell membrane. In the absence of NaCl, the relaxation of this aggregates accompanying the decrease in $DS_{ion}$ was not observed.

Increased unfrozen water imply inhibition of extracellular ice crystal formation, thus also positively affecting the cryoprotective effect. However, in the case of low-molecular-weight zwitterion, which is highly effective in suppressing ice crystal formation, the proportion of unfrozen water was 31%[1], which was larger than that of 10% (w/v) poly($VimC_3C$) $DS_{ion(50\%)}$ / 1% (w/v) NaCl aqueous solution (15%). In other word, changing the $DS_{ion}$ of polyZI to increase the proportion of unfrozen water does not have a critical effect on the cryoprotecting effect.

These results suggest that the strengthening of the extracellular matrix and the inhibition of extracellular ice crystal formation by lowering $DS_{ion}$ are not critical for cryopreservation using polyZI.

Table 3-3 The proportion of unfrozen water in the indicated solutions. In this study, poly($VimC_3C$) (28 kg/mol) is same poly($VimC_3C$) $DS_{ion(100\%)}$, unless noted. Material from: Kato, Y. *et al.*, Optimization of zwitterionic polymers for cell cryopreservation, *Macromolecular Bioscience*, [2024], [publisher - as it appears on our copyright page]'

| | The proportion of unfrozen water (%) |
|---|---|
| 10% (w/v) poly($VimC_3C$) (15 kg/mol) aqueous solution | 0 |
| 10% (w/v) poly($VimC_3C$) (28 kg/mol) aqueous solution | 0 |
| 10% (w/v) poly($VimC_3C$) (98 kg/mol) aqueous solution | 0 |
| 10% (w/v) poly($VimC_3C$) (15 kg/mol) / 1% (w/v) NaCl aqueous solution | 5 |
| 10% (w/v) poly($VimC_3C$) (28 kg/mol) / 1% (w/v) NaCl aqueous solution | 4 |
| 10% (w/v) poly($VimC_3C$) (98 kg/mol) / 1% (w/v) NaCl aqueous solution | 5 |
| 1% (w/v) NaCl aqueous solution | 13 |
| 10% (w/v) poly($VimC_3C$) $DS_{ion(10\%)}$ aqueous solution | 3 |

| | |
|---|---|
| 10% (w/v) poly($VimC_3C$) $DS_{ion(30\%)}$ aqueous solution | 6 |
| 10% (w/v) poly($VimC_3C$) $DS_{ion(50\%)}$ aqueous solution | 0 |
| 10% (w/v) poly($VimC_3C$) $DS_{ion(70\%)}$ aqueous solution | 4 |
| 10% (w/v) poly($VimC_3C$) $DS_{ion(100\%)}$ aqueous solution | 0 |
| 10% (w/v) poly($VimC_3C$) $DS_{ion(10\%)}$ / 1% (w/v) NaCl aqueous solution | 16 |
| 10% (w/v) poly($VimC_3C$) $DS_{ion(30\%)}$ / 1% (w/v) NaCl aqueous solution | 17 |
| 10% (w/v) poly($VimC_3C$) $DS_{ion(50\%)}$ / 1% (w/v) NaCl aqueous solution | 15 |
| 10% (w/v) poly($VimC_3C$) $DS_{ion(70\%)}$ / 1% (w/v) NaCl aqueous solution | 8 |
| 10% (w/v) poly($VimC_3C$) $DS_{ion(100\%)}$ / 1% (w/v) NaCl aqueous solution | 5 |
| 10% (w/v) poly($VpyC_3C$) $DS_{ion(40\%)}$ aqueous solution | 0 |
| 10% (w/v) poly($VpyC_3C$) $DS_{ion(50\%)}$ aqueous solution | 0 |
| 10% (w/v) poly($VpyC_3C$) $DS_{ion(100\%)}$ aqueous solution | 0 |
| 10% (w/v) poly($VpyC_3C$) $DS_{ion(40\%)}$ / 1% (w/v) NaCl aqueous solution | 13 |
| 10% (w/v) poly($VpyC_3C$) $DS_{ion(50\%)}$ / 1% (w/v) NaCl aqueous solution | 15 |
| 10% (w/v) poly($VpyC_3C$) $DS_{ion(100\%)}$ / 1% (w/v) NaCl aqueous solution | 3 |

I concluded that poly($VimC_3C$) was the best among those considered, and that its molecular weight and $DS_{ion}$ did not significantly affect its potential for cryoprotection.

**Washing Methods**

I investigated washing methods to effectively remove polyZI from cells after cryopreservation. Usually, the freezing medium (100 μL) was washed once by adding culture medium (1 mL) and centrifuging. More effective washing solvents were sought: medium, water, 0.5% (w/v) NaCl aqueous solution, 2% (w/v) NaCl aqueous solution, 3.1% (w/v) zwitterion monomer ($VimC_3C$) aqueous solution, 6.4% (w/v) sucrose aqueous solution and 6.4% (w/v) sucrose / 0.5% (w/v) NaCl aqueous solution. The zwitterion monomer aqueous solution and the sucrose aqueous solution were equimolar to the 1.0% (w/v) NaCl aqueous solution. I did not examine washing with the 1.0% (w/v) NaCl aqueous solution, because it has similar nature to the culture medium. The 6.4% (w/v) sucrose aqueous solution resulted in a high living cell number after freezing, thawing, and culturing (Fig. 3-26). Water and the NaCl aqueous solutions were not effective, whereas the zwitterion monomer ($VimC_3C$) aqueous solution was only effective for BOSC. Since NaCl specifically affects the polyZI aggregation states (see Fig. 3-10), neutral or zwitterionic solutions are probably better than NaCl solutions. In this way, washing with sucrose solution made it easier to remove polyZI and cells proliferated, but the complete removal was not achieved at this primitive stage. Since polyZI is a new cryoprotectant, I believe that with further study I will find a way to remove it.

I also assumed that since the polyZI is attached to cells, trypsinisation may detach it from the

cells; however, no positive effect was observed (see Fig. 3-26).

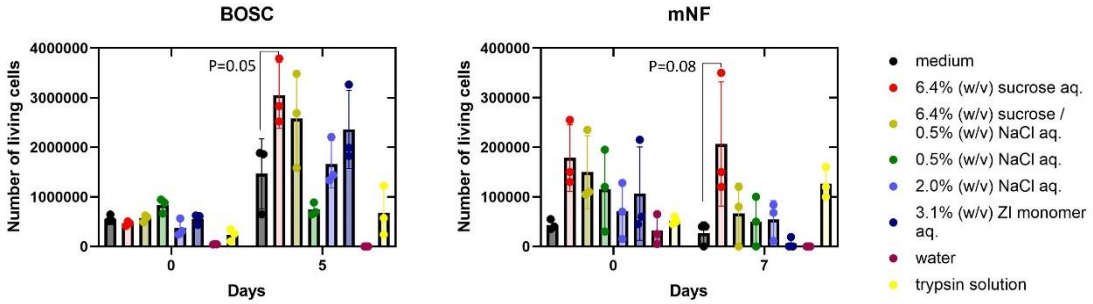


Fig. 3-26 Number of living BOSC cells and mNF immediately and 5–7 days after thawing and washing with the indicated solutions. Trypsin solution: cells were added to trypsin solution (0.5 w/v% trypsin-5.3 mmol/L EDTA・4Na solution without phenol red (×10), Fujifilm Wako Pure Chemical Corporation)) that had been diluted 10 times with phosphate buffered saline (PBS) and incubated for 3 min before centrifugation. There is no statistical significance between the samples ($p > 0.1$) ($n = 3$, biological triplicates, one-way ANOVA) Material from: Kato, Y. *et al.*, Cell-compatible isotonic freezing media enabled by thermo-responsive osmolyte-adsorption/exclusion polymer matrices, *Communications Chemistry*, [2023], [publisher - as it appears on our copyright page]'

**Concentrations**

The polyZI concentration was investigated, which is thought to affect the cryoprotecting effect. Varying concentrations of poly(VimC$_3$C)_DS$_{ion(100\%)}$ from 1% to 30% (w/v), 10%, or 20% (w/v) gave the highest living cell number immediately post-thaw (Fig. 3-27 (a)), while 10% was applied in the above experiments. I thought that lowering the polyZI concentration in the solution would make it easier to remove polyZI and allow cells to proliferate. However, after 5 days from thawing, the cell numbers given with 20% (w/v) poly(VimC$_3$C) aqueous solution were the highest (Fig. 3-27 (b)). In contrast, 10% (w/v) poly(VimC$_3$C) produced a lower yield.

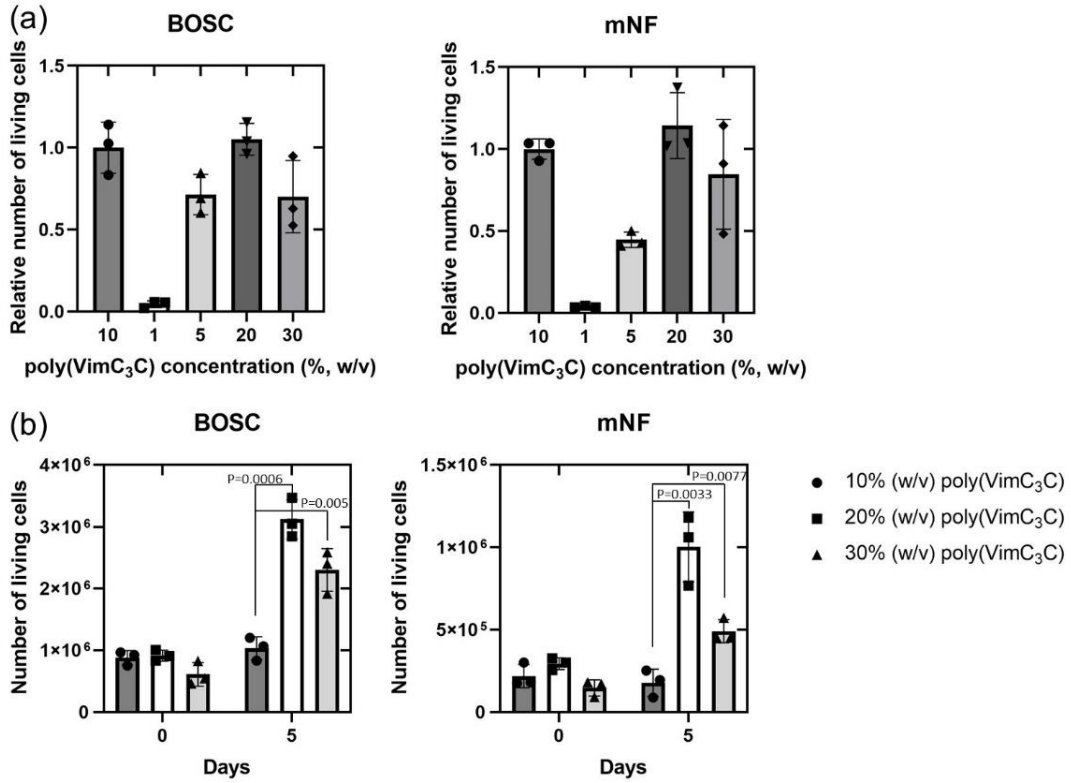


Fig. 3-27 (a) The relative number of living BOSC and mNF cells immediately post-thaw after cryopreservation using poly($VimC_3C$) with various concentrations. (b) Number of living BOSC and mNF cells 0 and 5 days post-thaw after cryopreservation, using poly($VimC_3C$) with various concentrations. Original solution developed in the previous study is 10% (w/v) poly($VimC_3C$)_$DS_{ion(100\%)}$ / 1% (w/v) NaCl aqueous solution DSion: 100%. (n = 3, biological triplicates, one-way ANOVA) Material from: Kato, Y. *et al.*, Optimization of zwitterionic polymers for cell cryopreservation, *Macromolecular Bioscience*, [2024], [publisher - as it appears on our copyright page]'

It has been previously reported that polyZI strongly interacts with the cell membrane[12]and is difficult to remove by washing after cryopreservation, resulting in low proliferation[7]. Although lowering the polyZI concentration generally seemed to facilitate polyZI removal, 20% (w/v) was unexpectedly better than 10% (w/v) (Fig. 3-27). This may be due to a shift in the aggregation state of poly($VimC_3C$) in aqueous solution, depending on the poly($VimC_3C$) concentration. 10% (w/v) poly($VimC_3C$) partly aggregates in pure water, and the addition of NaCl partially relaxes the aggregation[7]. Because the aggregation suppress the interaction to water, the aggregation states of poly($VimC_3C$) partly can be discussed by shift in osmotic pressure. Since the osmotic pressure did not increase with the addition of 10% (w/v) poly($VimC_3C$) [osmotic pressure:327 mOsm vs. 289 mOsm for 1% (w/v) NaCl aqueous solution and 10% (w/v) poly($VimC_3C$) / 1% (w/v) NaCl aqueous solution,

respectively, Table 3-4], poly($VimC_3C$) does not strongly interact with water; indicating strong aggregation. In contrast, 20% (w/v) poly($VimC_3C$) increased the osmotic pressure [osmotic pressure:327 vs. 419 mOsm for 1% (w/v) NaCl aqueous solution and 20% (w/v) poly($VimC_3C$) / 1% (w/v) NaCl aqueous solution, respectively, Fig. 3-28]. These findings suggest that poly($VimC_3C$) relaxes when the concentration is 20% (w/v). The increase in osmotic pressure with increasing poly($VimC_3C$) concentration was found, even in the absence of NaCl. These results interestingly indicates self-relaxation of poly($VimC_3C$) [osmotic pressure:5 and 95 mOsm for 10 and 20% (w/v) poly($VimC_3C$) aqueous solution, respectively]. Good dispersion of poly($VimC_3C$) in 20% (w/v) poly($VimC_3C$) aqueous solution may explain the poly($VimC_3C$) removal, resulting in a high proliferation rate.

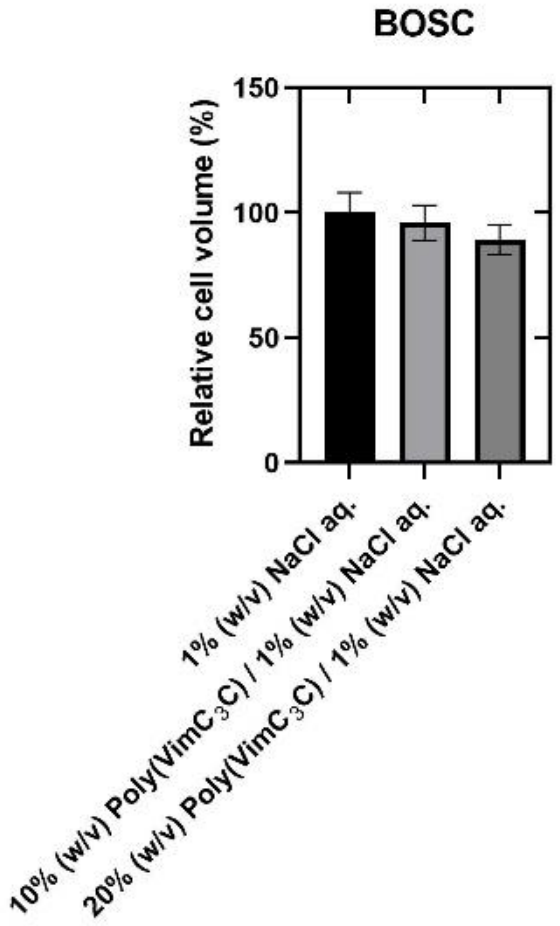


Fig. 3-28 The relative cell volume in poly($VimC_3C$) solutions with different concentrations after 60 min incubation. $DS_{ion}$: 100%. There is no statistical significance between the samples ($p > 0.1$) [p value of cell volume in solution between including polymer and not is 0.55 (10% (w/v) poly($VimC_3C$)), 0.14 (20% (w/v) poly($VimC_3C$))]. (n = 3, biological triplicates) Material from: Kato, Y. *et al.*, Optimization of zwitterionic polymers for cell cryopreservation, *Macromolecular Bioscience*, [2024],

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Table 3-4 The proportion of unfrozen water and osmotic pressure in the indicated solutions. In this study, poly($VimC_3C$) (28 kg/mol) is same poly($VimC_3C$) $DS_{ion(100\%)}$, unless noted. $DS_{ion}$: 100%. Material from: Kato, Y. *et al.*, Optimization of zwitterionic polymers for cell cryopreservation, *Macromolecular Bioscience*, [2024], [publisher - as it appears on our copyright page]'

| | The proportion of unfrozen water (%) | Osmotic pressure (mOsm) |
|---|---|---|
| 5% (w/v) poly($VimC_3C$) aqueous solution | - [a] | 3 |
| 10% (w/v) poly($VimC_3C$) aqueous solution | 0 | 5 |
| 20% (w/v) poly($VimC_3C$) aqueous solution | - [a] | 95 |
| 5% (w/v) poly($VimC_3C$) / 1% (w/v) NaCl aqueous solution | 5 | - [a] |
| 10% (w/v) poly($VimC_3C$) / 1% (w/v) NaCl aqueous solution | 9 | 289 |
| 20% (w/v) poly($VimC_3C$) / 1% (w/v) NaCl aqueous solution | 12 | 419 |
| 1% (w/v) NaCl aqueous solution | 11 | 327 |

[a] *not measured.*

10% (w/v) poly($VimC_3C$)_$DS_{ion(100\%)}$ / 1% (w/v) NaCl aqueous solution was almost isotonic in cell culture medium (~300 mOsm). This is an advantage for cryopreservation because typical freezing media cause osmotic shock[7]. I investigated osmotic shock in one of the optimized solutions [20% (w/v) poly($VimC_3C$)_$DS_{ion(100\%)}$ / 1% (w/v) NaCl aqueous solution]. It had a slightly higher osmotic pressure (419 mOsm; see Table 3-4). To determine the degree of osmotic shock, the cells were immersed in the solution for 60 min; and the cells did not shrink significantly (Fig. 3-28), indicating a negligible osmotic shock. This may be related to the features of polyZIs; the matrix of poly($VimC_3C$) has been found to be capable of adsorbing NaCl[24], thereby mitigating osmotic shock.

If the poly($VimC_3C$) matrix surrounds the cells, the permeability of the cell membrane can change. Cell expansion was investigated after the addition of the cell-permeable substance, DMSO. The expansion rate of cell volume decreased in the presence of the poly($VimC_3C$) matrix (Fig. 3-29). This suggests that the poly($VimC_3C$) matrix suppressed the influx of extracellular substances through free diffusion. This effect was not observed in 10% (w/v) poly($VimC_3C$)_$DS_{ion(100\%)}$ / 1% (w/v) NaCl aq. and therefore the firm matrix in the 20% solution may be a critical factor of the optimization, because the firm matrix can prevent the influx of ice nuclei. The formation of matrix takes around 30 min after addition of poly($VimC_3C$) solutions (Fig. 3-30).

In summary of the role of poly($VimC_3C$), although 20% (w/v) poly($VimC_3C$)_$DS_{ion(100\%)}$ / 1% (w/v) NaCl aqueous solution was slightly hypertonic and poly($VimC_3C$) did not cause osmotic shock, and the poly($VimC_3C$) matrix is assumed to prevent the influx of ice nuclei.

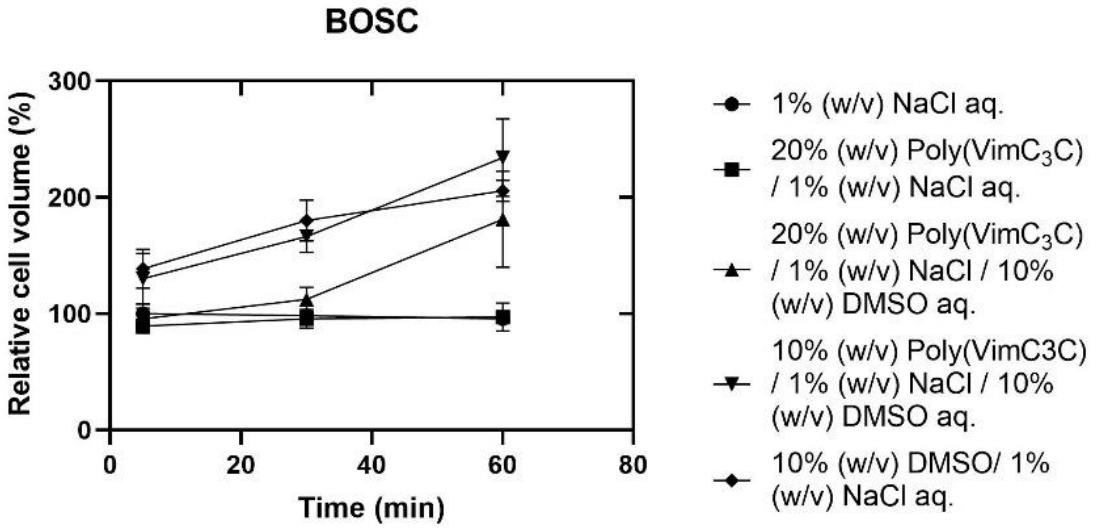



Fig. 3-29 The relative ell volume in the indicated solutions. $DS_{ion}$: 100%.

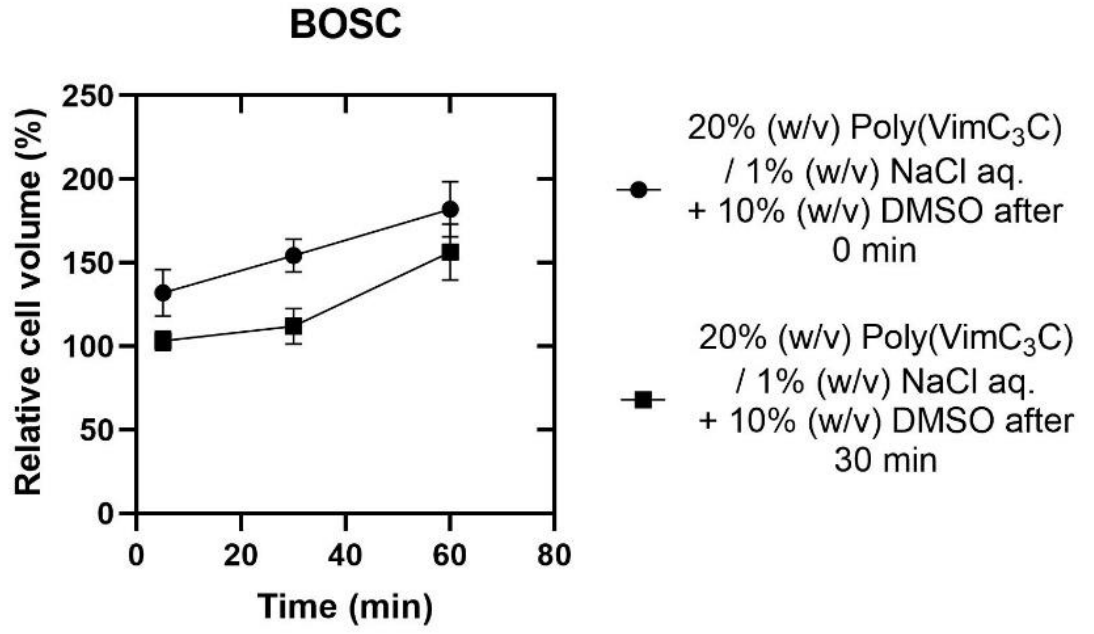



Fig. 3-30 The relative ell volume of the indicated solutions. Cells were incubated in 20% (w/v) poly($VimC_3C$) / 1% (w/v) NaCl aq. for 0 and 30 min, and then 10% (w/v) DMSO were added. $DS_{ion}$: 100%.

3.12 Cell proliferation after cryopreservation

Cell proliferation of mNF and BOSC were counted after cryopreservation using the optimized polyZI solution and washing with sucrose aqueous solution. Thawing with sucrose aqueous solution slightly increased the cell viability. Although there was no difference in the cell viability immediately after thawing with a sucrose aqueous solution at each concentration, the number of living cells after 5 days of culture decreased when 10% (w/v) poly($VimC_3C$) / 1% (w/v) NaCl aqueous solution was used and increased the most when 20% (w/v) poly($VimC_3C$) / 1% (w/v) NaCl aqueous solution was used, indicating performance equivalent to that of the commercial (Fig. 3-31). The cryoprotective effect and cell proliferation after cryopreservation were improved using the optimized solutions (20% (w/v) poly($VimC_3C$) / 1% (w/v) NaCl aqueous solution) in the other cells, which was unsuccessful in the previous article[7].

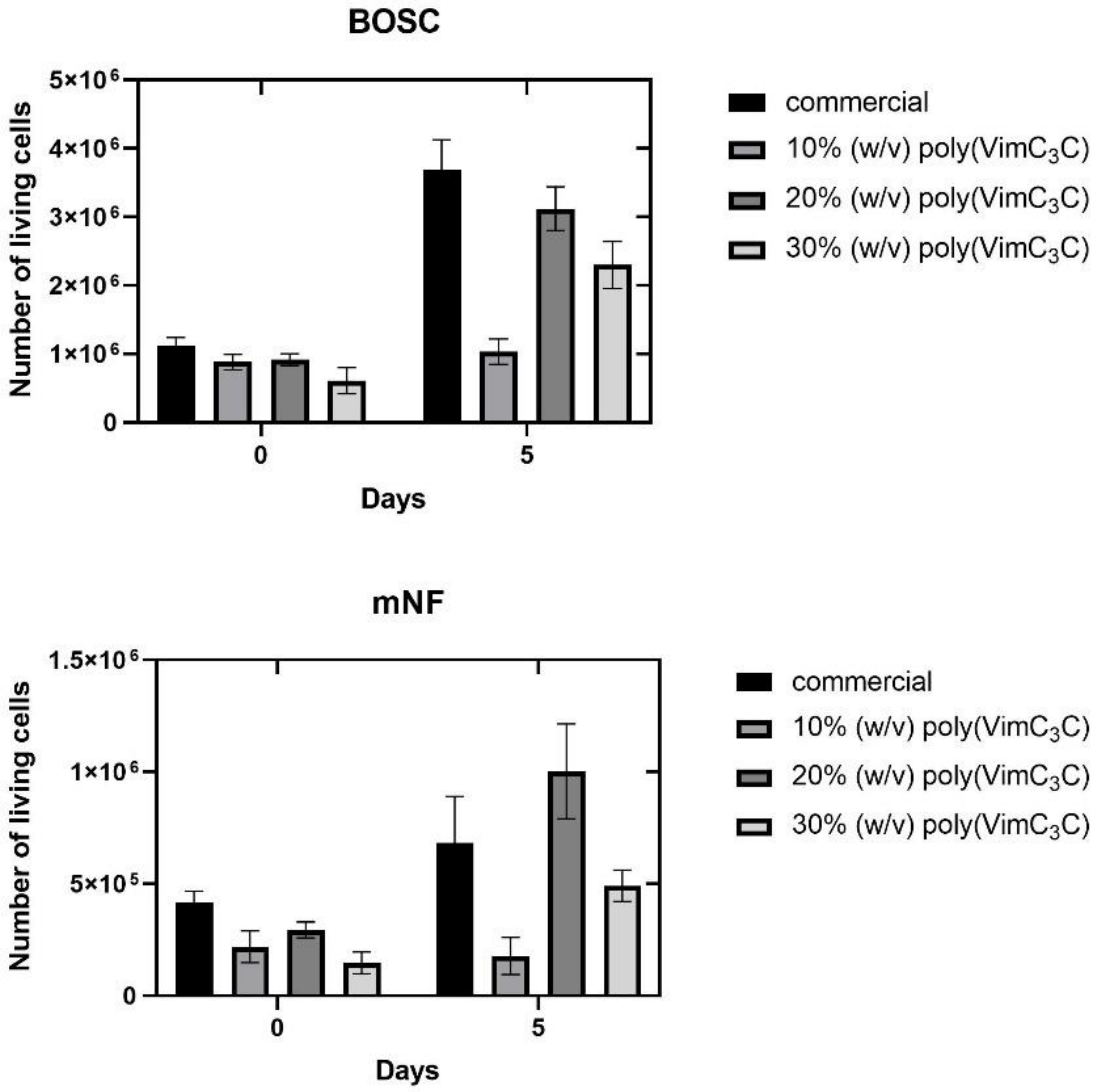


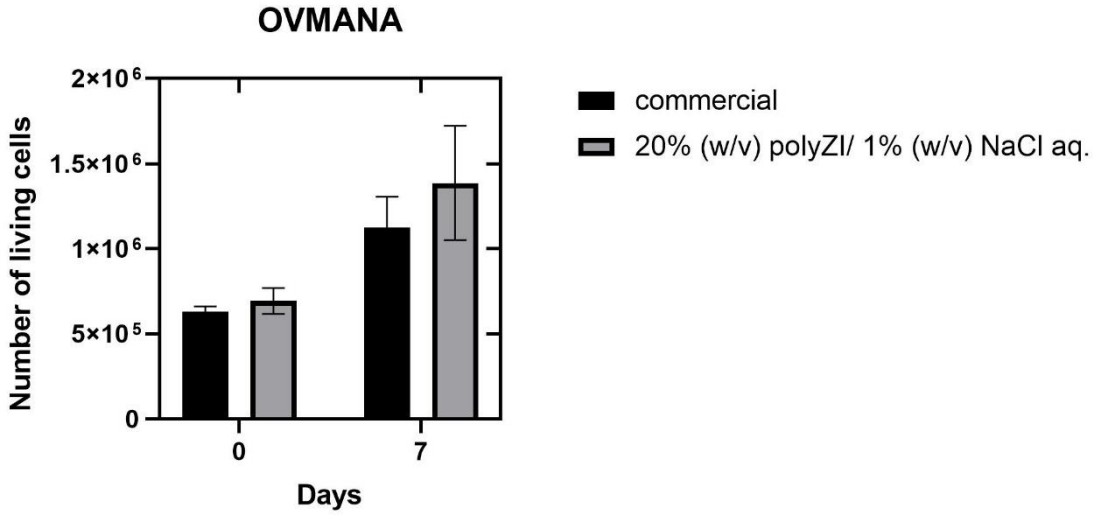

Fig. 3-31 Number of living BOSC and mNF cells 0 and 5 days post-thaw after cryopreservation, using

the optimized polyZI (20% (w/v) poly($VimC_3C$)) aqueous solution and washing with sucrose aqueous solution. (n = 3, biological triplicates, one-way ANOVA)

### 3.13 Cryopreservation freezing-vulnerable cells

Freeze-vulnerable cells (OVMANA, Vn1919 and K562) were subjected to cryopreservation using the polyZI/NaCl aqueous solution. As a result, the relative number of living cells immediately after thawing tends to be higher than that when using a commercial medium, suggesting the importance of isotonic cryopreservation (Fig. 3-32). The relative number of living OVMANA cells after 7 days of culture was 2.3 times higher than when using a commercial (Fig. 3-32). On the other hand, the relative number of living Vn1919 and K562 cells immediately after thawing was also slightly higher when using the commercial, but the number of living Vn1919 and K562 cells after 7 days of culture was almost the same as when using the commercial.

The characteristics of polyZI as a cryoprotectant are that it inhibits the influx of ice crystals and alleviates osmotic damage. This suggests that these two mechanisms are not the reason why Vn1919 and K562 cannot be efficiently cryopreserved. Because the mechanisms that make cryopreservation difficult differ for each cell, it may be difficult to develop a cryoprotectant that can cryopreserve all cells. However, polyZI seems to have made it possible to efficiently cryopreserve cells that are otherwise difficult to cryopreserve due to the influx of ice crystals or osmotic shock. These results suggested that a cryoprotectant using polyZI could be an effective cryopreserve for cells such as OVMANA that are difficult to cryopreserve.



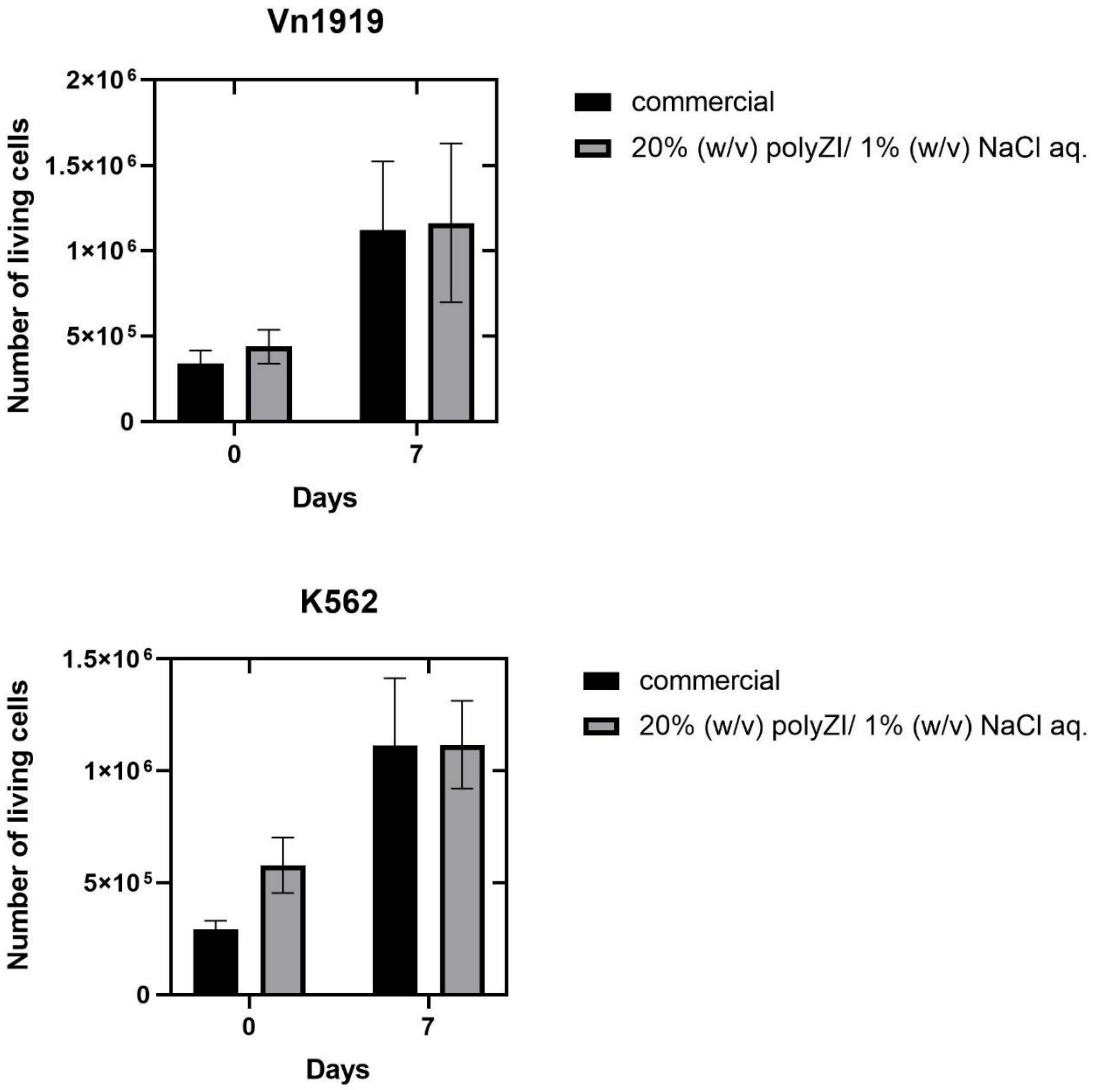



Fig. 3-32 Number of living OVMANA, Vn1919 and K562 cells 0 and 5 days post-thaw after cryopreservation, using the optimized polyZI (20% (w/v) poly($VimC_3C$)) aqueous solution and washing with sucrose aqueous solution. (n = 3, biological triplicates, one-way ANOVA)

3.14 Conclusion

By combining polyZI with NaCl, I was able to develop a new isotonic freezing medium. The freezing medium using polyZI is isotonic and therefore has very low toxicity. Furthermore, the presence of NaCl in polyZI suppresses intramolecular interactions between polymers, making it possible to suppress aggregation. Furthermore, when polyZI and NaCl are combined, their respective charges interact strongly, suggesting that polyZI and NaCl aggregate locally around the cell membrane to form a matrix. It was suggested that the high cryoprotecting effect of polyZI is largely related to the effect of the matrix on suppressing the inflow of extracellular ice crystals into cells.

It was reported that the polyZI matrix is difficult to remove after thawing, thereby inhibiting cell proliferation after thawing. However, when the concentration of polyZI was increased to 20% (w/v) , the aggregation of polyZI in water was alleviated, polyZI could be efficiently removed, and a high cell proliferation rate was obtained.

The optimal structure of polyZI was the imidazolium/carbonate system, and molecular weight and degree of ionic substitution did not have a strong relationship with the cryoprotecting effect. This isotonic freezing medium, 20% (w/v) poly($VimC_3C$) / 1.0% (w/v) NaCl aqueous solution, showed cryoprotecting effects equivalent to commercial. This freezing medium was able to cryopreserve even freezing-vulnerable cells without the use of cell-permeable cryoprotectants such as DMSO.

## 3.15 Reference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# 4. Conclusion

## 4.1 Conclusion

In this study, I describe the development of new cell freezing media using zwitterions. I have developed new cryoprotectants to cryopreserve freezing-vulnerable cells. At this time, I focused on zwitterion, which allows us to freely design the structure of molecules. I discovered the cryoprotecting mechanism of zwitterion and optimized a better cryoprotectant by investigating its interactions with water, molecules, and cell membranes. By using zwitterion as a cryoprotectant, I demonstrated high cryoprotecting effects even when cryopreserving various cells. Furthermore, it has become possible to cryopreserve freezing-vulnerable cells. No problems were observed in proliferation after cryopreservation, and no changes in cell properties occurred due to cryopreservation. In addition, investigating the cryoprotecting mechanism of zwitterion provided insight into the cryoprotecting mechanism, which is important for cell cryopreservation.

In Chapter 2, I cryopreserved various cells using zwitterion, measured the cryoprotecting effect of zwitterion, and investigated the cryoprotecting mechanism from outside the cell. There was no correlation between zwitterion structure, vitrification, the proportion of unfrozen water, osmotic pressure, and cell viability. Zwitterions showed high cryoprotecting effects due to their strong inhibition ice crystal formation, dehydration ability, and low toxicity. In particular, since zwitterions have large molecular weight and are ionic, it was found to act as non-cell-permeable-cryoprotectants, and intracellular dehydration was important. In order to further improve the cryoprotecting effect of zwitterions, DMSO was added as a cell-permeable cryoprotectant, which showed a high cryoprotecting effect on various cells. In particular, even if freezing-vulnerable cells were cryopreserved more efficiently when using zwitterion/DMSO than using commercial, and proliferated as well after cryopreservation as before cryopreservation. Interestingly, zwitterions were found to protect cells from the harmful effects of DMSO, so even when mixed with DMSO, a high cryoprotecting effect was obtained with low toxicity.

In Chapter 3, various cells were cryopreserved using polymerized zwitterion (polyZI), and the cryoprotecting effect of polyZI, and the cryoprotecting mechanism of polyZI was investigated. PolyZI has very strong intramolecular interactions and aggregates in aqueous solution, but the addition of NaCl alleviates the aggregation. polyZI forms a matrix around the cell membrane and prevents the influx of ice crystals from outside the cell. This made it possible to develop cryoprotectants that do not require DMSO, a cell-permeable cryoprotectant that is toxic to cells. Interestingly, the polyZI matrix had the unique property of adsorbing and desorbing NaCl depending on temperature and controlling osmotic pressure, making it suitable for cryopreservation. Through these mechanisms, cells can be cryopreserved without applying strong osmotic stress, and cryoprotecting effects have been demonstrated on a variety of cells. By optimizing the concentration of polyZI, I was able to control the intramolecular interactions of polyZI in solution. This enabled efficient removal of polyZI

after cryopreservation, making cryopreservation possible without interfering with cell proliferation.

Cryopreservation technology is essential in research using cells. Therefore, the development of freezing media has been going on for decades. In order to solve the problems of conventional cryoprotectants, I developed cryopreservation using a new compound, zwitterions. Based on the results obtained so far, zwitterion enables the cryopreservation of various cells and enables the discovery of a new cryoprotecting mechanism. By optimizing the interactions with water, substances, and cell membranes, I was able to obtain a cryoprotecting effect that exceeds that of commercial. In addition, there are still many unknowns, such as the cryoprotecting mechanism being completely different depending on the structure and polymerization of zwitterion, so it is thought that future research will advance the development of zwitterion as a new cryoprotectant. I established guidelines for the development of zwitterion-based cryoprotectants.

## 4.2 Future development

I cryopreserved cells using zwitterion for the first time. In this study, zwitterions, new cryoprotectants, were confirmed to have a cryoprotecting effect that is superior to that of commercial. From this, zwitterion can be considered as an alternative to FBS as the same non-cell-permeable cryoprotectant. Because FBS contains many unknown substances, it is unclear which substances affected the results, making it impossible to obtain accurate experimental data. Furthermore, FBS can only be obtained from cow fetuses, is extremely expensive, and is considered an animal welfare issue. Currently, proteins that can replace serum are synthesized using microorganisms, but there is a risk that viruses may be contaminated during synthesis. However, since zwitterion can be manufactured through chemical synthesis, there is no risk of virus contamination, and once a production line is established, the costs of freezing media can be reduced. From this, zwitterion has the potential to be marketed as a new low-cost, high-performance freezing medium.

In this study, I was only able to measure the cell viability of some cells. In the future, zwitterions may become useful as cryoprotectants for undifferentiated cells. Common commercial freezing media include DMSO. However, DMSO has been reported to promote differentiation. In the case of polyZI, it shows a high cryoprotecting effect even without DMSO, so it is likely to be useful as a cryoprotectant that maintains an undifferentiated state. In addition, there is a need for freezing media for things other than cells, such as spheroids, tissues, and organs, so it is believed that zwitterions have the potential to become useful cryoprotectants if further research is conducted into applications other than cells.

In this study, I was only able to measure the survival rate of some cells. In the future, zwitterions may become useful as cryoprotectants for undifferentiated cells. Common commercial freezing media include DMSO. However, DMSO has been reported to promote differentiation. In the case of polyZI, it shows a high cryoprotecting effect even without DMSO, so it is likely to be useful as a cryoprotectant that maintains an undifferentiated state. In addition, there is a need for freezing media for things other

than cells, such as spheroids, tissues, and organs, so it is believed that zwitterions have the potential to become useful cryoprotectants if further research is conducted into applications other than cells.

Therefore, research using zwitterions is expected to have a major impact on the cryopreservation field, as well as the establishment of new preservation methods.

4.3 List of publications

**Original papers**

・Title：Synthetic zwitterions as efficient non-permeable cryoprotectants
Author's names：Yui Kato, Takuya Uto, Daisuke Tanaka, Kojiro Ishibashi, Akiko Kobayashi, Masaharu Hazawa, Richard W. Wong, Kazuaki Ninomiya, Kenji Takahashi1, Eishu Hirata, Kosuke Kuroda
Journal title：Commun. Chem.
Volume, pages and the published year and month：4, 151 and 2021

・ Title ： Cell-compatible isotonic freezing media enabled by thermo-responsive osmolyte-adsorption/exclusion polymer matrices
Author's names：Yui Kato, Yuya Matsuda, Takuya Uto, Daisuke Tanaka, Kojiro Ishibashi, Takeru Ishizaki, Akio Ohta, Akiko Kobayashi, Masaharu Hazawa, Richard W. Wong, Kazuaki Ninomiya, Kenji Takahashi, Eishu Hirata, Kosuke Kuroda
Journal title：Commun. Chem.
Volume, pages and the published year and month：6, 260 and 2023

・Title：Optimization of zwitterionic polymers for cell cryopreservation
Author's names：Yui Kato, Takeru Ishizaki, Daisuke Tanaka, Kojiro Ishibashi, Yuya Matsuda, Akiko Kobayashi, Masaharu Hazawa, Richard W. Wong, Kenji Takahashi, Eishu Hirata, Kosuke Kuroda
Journal title：Macromolecular Bioscience.
Volume, pages and the published year and month：

・Title：Characterization and Application of Carboxylate-type zwitterions synthesized by one-step
Author's names：T. Komori, Y. Kato, K. Ishibashi, K. Ninomiya, N. Wada, D. Hirose, K. Takahashi, E. Hirata, K. Kuroda
Journal title：J. Ion. Liq.
Volume, pages and the published year and month：2, 100027 and 2022

・Title：Synthesis of a cellulose dissolving liquid zwitterion from general and low-cost reagents
Author's names：G. Sharma, Y. Kato, A. Hachisu, K. Ishibashi, K. Ninomiya, K. Takahashi, E. Hirata,

K. Kuroda
Journal title：Cellulose
Volume, pages and the published year and month：29, 3017 and 2022

・Title：Non-aqueous, zwitterionic solvent as an alternative for dimethyl sulfoxide in the life sciences
Author’s names：K. Kuroda, T. Komori, K. Ishibashi, T. Uto, I. Kobayashi, R. Kadokawa, Y. Kato, K. Ninomiya, K. Takahashi, E. Hirata
Journal title：Commun. Chem.
Volume, pages and the published year and month：3, 163 and 2020

**Other achievement**

・Shibuya Academic, Cultural and Sports Promotion Foundation Award, November 2020
・Green Chemistry Award (Poster Award), 10th Ionic Liquid Symposium, November 2019
・Kanazawa University President's Award, March 2022 (at the time of completion of master's course)

4.4 Acknowledgment

This paper is a summary of research conducted at the Biochemical Engineering Laboratory, Kanazawa University. I would like to thank a variety of people for allowing me to advance my research step by step with the cooperation of various people.

I have received great guidance from Associate Professor Kosuke Kuroda ever since I was assigned to his laboratory. At first, I didn't understand anything, but he provided me with a lot of guidance, from the idea of the research to the research and writing the paper, and thanks to his advice, I was able to proceed with the research smoothly. When the experiment doesn't go well and I was worried, we discussed ways to find a way out, and he listened carefully to my suggestions and gave their opinions. Thanks to this, I had the opportunity to learn about the significance and enjoyment of research. He taught me every step of the way about things that come naturally to me as a researcher, and he also passionately taught me how to give presentations that people could understand. I am also very encouraged by the fact that I was able to write the paper that was my goal in graduate school. Although it was difficult to write, I was able to put it together thanks to his many corrections and guidance in the process. I am very grateful. I hope that Kuroda laboratory will become an even better lab in the future.

We would like to express our gratitude to Associate Professor Eishu Hirata and Assistant Professor Kojiro Ishibashi of the Kanazawa University Institute for Cancer Progression Control, who carefully taught us how to conduct biological experiments from the beginning. When conducting experiments, they gave me many suggestions and advice, and allowed me to use various devices. Thank you for giving me easy-to-understand guidance even though I have no knowledge of biology. I

would like to once again thank Professor Kenji Takahashi for kindly lending me his equipment despite being a member of the Kuroda laboratory.

My senior colleagues patiently taught me how to use the equipment and how to synthesize. My junior colleagues helped me with the experiment. I was very inspired by seeing everyone working so hard on their research. Also, everyone talked to me in a friendly manner in my daily life, and I was able to spend a very fulfilling day. I have learned a lot from all of you and I am very grateful. I wish you all the best in your future endeavors.

Finally, I would like to express my gratitude to my parents who supported me both physically and mentally. Entering graduate school was very meaningful for me. I was able to advance to this university because I was able to noticeably improve not only my research basics, but also my writing skills and logical thinking skills. It's still a long way off, but I want to make sure that I can support my parents in the future.