BIOMIMETIC ICE-INHIBITING MATERIAL AND CRYOPRESERVATION SOLUTION COMPRISING SAME

The present application claims priority to Chinese Patent Nos. 2019102824189, 2019102824225, 2019102824174, 201910282416X and 2019102819867 filed with the China National Intellectual Property Administration on April 9, 2019, which are incorporated herein by reference in their entirety.

TECHNICAL FIELD

The present invention relates to the technical field of biomedical materials, and particularly to a biomimetic ice growth inhibition material and a cryopreservation solution comprising the same.

BACKGROUND

Cryopreservation is to store a biological material at an ultra-low temperature to slow down or stop cell metabolism and division, and to continue development once normal physiological temperature is recovered. Since its advent, this technology has become one of indispensable research methods in the field of natural science, and has been widely adopted. In recent years, with the increasing pressure of life, human fertility tends to decline year by year, and thus fertility preservation is more and more emphasized. The cryopreservation of human germ cells (sperms and oocytes), gonad tissues and the like has become an important means of fertility preservation. In addition, as the world population ages, the need for cryopreservation of donated human cells, tissues or organs that can be used for regenerative medicine and organ transplantation is growing fast. Therefore, how to efficiently cryopreserve precious cells, tissues and organ resources for unexpected needs has become a scientific and technical problem to be solved urgently.

Currently, the most commonly available cryopreservation method is vitrification. The vitrification technology adopts a permeable or impermeable cryoprotectant. Although liquid inside and outside a cell can be directly vitrified in the rapid freezing process so as to avoid the damage resulting from the formation of ice crystals in the freezing process, cryopreservation reagents of the prior art are not effective in controlling the growth of the ice crystals in the rewarming process and thus damage the cell. Because the ice growth inhibition mechanisms of antifreeze proteins and biomimetic ice growth inhibition materials at the molecular level is still controversial, the research and development of the biomimetic ice growth inhibition materials has to rely on “trial and error” to test the ice growth inhibition effect of a certain ice growth inhibition material, which is characterized by a heavy workload, and a low probability of success. Currently, commonly available cryopreservation reagents have the problems of having no

capability of effectively controlling the growth of ice crystals in the rewarming process and being high in toxicity.

SUMMARY

In order to overcome the defects in the prior art, the present invention provides a molecular design method for a biomimetic ice growth inhibition material and a screening method for an ice growth inhibition material, which can guide people to purposefully synthesize and screen the biomimetic ice growth inhibition material. The present invention also provides a biomimetic ice growth inhibition material obtained based on the method and a cryopreservation reagent comprising the same.

The present invention provides the following technical solutions:

In a first aspect of the present invention, a molecular design method for an ice growth inhibition material is provided, comprising the following steps:

(1) constructing a library for structures of compound molecules, wherein the compound molecules comprise a hydrophilic group and an ice-philic group;

(2) simulating and evaluating the spreading performance of each of the compound molecules at an ice-water interface by adopting molecular dynamics (MD) simulation; and

(3) screening the ice growth inhibition molecules with desired affinities for ice and water according to the step (2).

According to the present invention, the main chain of the ice growth inhibition molecule is a carbon chain or a peptide chain.

According to the present invention, the hydrophilic group is a functional group capable of forming a non-covalent interaction with a water molecule, for example, forming a hydrogen bond, a Van der Waals interaction, an electrostatic interaction, a hydrophobic interaction or a π-π interaction with water; illustratively, the hydrophilic group may be selected from at least one of hydroxyl (-OH), amino (-NH_2), carboxyl (-COOH), amido (-$CONH_2$), and the like, or, for example, from a compound molecule, such as a hydrophilic amino acid such as proline (L-Pro), arginine (L-Arg) and lysine (L-Lys), glucono delta-lactone (GDL) and a saccharide, and a molecular fragment thereof.

According to the present invention, the ice-philic group is a functional group capable of forming a non-covalent interaction with ice, for example, forming a hydrogen bond, a Van der Waals interaction, an electrostatic interaction, a hydrophobic interaction or a π-π interaction with ice; illustratively, the ice-philic group may be selected from hydroxyl (-OH), amino (-NH_2), phenyl (-C_6H_5) and pyrrolidinyl (-C_4H_8N), or, for example, from a compound molecule, such as an ice-philic amino acid such as glutamine (L-Gln), threonine (L-Thr) and aspartic acid (L-Asn), a

benzene ring (C_6H_6) and pyrrolidine (C_4H_9N), and a molecular fragment thereof.

According to the present invention, the ice growth inhibition material may be formed by bonding a hydrophilic group and an ice-philic group through a covalent bond.

According to the present invention, the ice growth inhibition material may be formed from a hydrophilic group and an ice-philic group through a non-covalent bond such as an ionic interaction.

According to the present invention, the method further comprises a step (4): a step of synthesizing the ice growth inhibition molecule (ice growth inhibition material), for example, by a known chemical synthesis method such as a polymerization reaction, a condensation reaction, or a biological fermentation method of genetically engineered bacteria.

The present invention also provides an ice growth inhibition material obtained by the molecular design method according to the first aspect.

In a second aspect of the present invention, a method for screening an ice growth inhibition material is provided, comprising the following steps: (a) determining the affinity of an ice growth inhibition material for water; and (b) determining the spreading performance of the ice growth inhibition material at an ice-water interface.

As an embodiment of the present invention, the step (a) may be achieved by methods such as determining a solubility, a hydration constant, a dispersion size and a diffusion coefficient of the ice growth inhibition material in water, and/or calculating a number of the intermolecular hydrogen bonds formed between the ice growth inhibition material and water molecules; specifically, the number of the intermolecular hydrogen bonds formed between the ice growth inhibition material and water molecules are determined using MD simulation, or the dispersion size of the ice growth inhibition material in an aqueous solution is determined using dynamic light scattering.

As an embodiment of the present invention, the step (b) may be achieved by determining the amount of the ice growth inhibition material adsorbed on an ice surface at an ice-water interface, and/or the affinity of the ice growth inhibition material for ice may be determined by calculating the number of the intermolecular hydrogen bonds formed between the ice growth inhibition material and ice-water molecules; specifically, the number of the intermolecular hydrogen bonds formed between the ice growth inhibition molecules and ice-water molecules is determined, for example, by MD simulation, or the amount of the ice growth inhibition material molecules adsorbed on an ice surface is determined at an ice-water interface by an ice adsorption experiment.

According to the present invention, the ice adsorption experiment comprises determining the amount of the ice growth inhibition material adsorbed on an ice surface.

According to the present invention, the amount of the ice growth inhibition material adsorbed on

an ice surface = (the mass of the ice growth inhibition material adsorbed on the ice surface m_1/total mass of the ice growth inhibition material in a stock solution comprising the ice growth inhibition material m_2) × 100%.

As an embodiment of the present invention, the ice adsorption experiment comprises the following steps:

S1, taking an ice growth inhibition material of the mass m_2 to prepare an aqueous solution of the ice growth inhibition material, and cooling to a supercooling temperature;

S2, placing a pre-cooled temperature-regulating rod into the aqueous solution to induce the growth of an ice layer on the surface of the temperature-regulating rod, continuously stirring the aqueous solution to enable the ice growth inhibition material to be gradually adsorbed onto the surface of the ice layer, and keeping the temperature of the aqueous solution and the temperature-regulating rod at a supercooling temperature; and

S3, determining the amount m_1 of the ice growth inhibition material adsorbed on the ice surface.

According to an embodiment of the present invention, the temperature-regulating rod is a rod-shaped object made of a thermally conductive material. The rod-shaped object may be solid or hollow. When the temperature-regulating rod is hollow, its hollow inner cavity is provided with a cooling liquid to flow, and the temperature of the temperature-regulating rod can be regulated by adjusting the temperature of the cooling liquid, thereby controlling growth speed of an ice block.

According to an embodiment of the present invention, the temperature-regulating rod can be pre-cooled in one of the modes of freezing by liquid nitrogen, dry ice, an ultralow temperature refrigerator and the like.

According to an embodiment of the present invention, the supercooling degree and the adsorption time are maintained unchanged during the ice adsorption experiment, so that the surface area of the ice formed on the surface of the temperature-regulating rod is maintained unchanged within an allowable error range.

According to an embodiment of the present invention, aqueous solutions of an ice growth inhibition material with different concentrations are prepared to carry out an ice adsorption experiment, so that the applicable concentration ranges of the same ice growth inhibition material in specific applications can be evaluated.

According to an embodiment of the present invention, the ice growth inhibition material in the step S1 may be fluorescently pre-labeled, for example, with fluorescein, and the fluorescein may be selected from at least one of fluorescein isothiocyanate (FITC), tetraethylrhodamine (RB200), tetramethylrhodamine isothiocyanate (TRITC), propidium iodide (PI), and the like. It will be appreciated by those skilled in the art that the fluorescent label functions to determine the amount

of the ice growth inhibition material, and thus, if the amount of the ice growth inhibition material adsorbed can be accurately measured by other means, or the material itself has absorption characteristics in an ultraviolet or fluorescent spectrum, no fluorescent label is required.

According to an embodiment of the present invention, the step S3 comprises:

S3a, taking out the ice block after adsorption, rinsing the ice surfaces with purified water, and melting the ice block to give an adsorption solution of the ice growth inhibition material; and

S3b, determining the volume V of the melted adsorption solution of the ice growth inhibition material, determining the mass/volume concentration c of the ice growth inhibition material in the adsorption solution, and calculating the mass m_1 of the ice growth inhibition material adsorbed on the ice surface through the formula $m_1 = cV$.

According to an embodiment of the present invention, in the S3b, the concentration c can be determined by a method known in the art, such as ultraviolet-visible spectroscopy and fluorescence spectroscopy.

According to the present invention, the method is used for screening a material for inhibiting the growth of ice crystals, such as a PVA, a polyamino acid, an antifreeze protein and a polypeptide.

According to the present invention, after the step (a) and/or the step (b), the method further comprises a step (c): evaluating the affinity of the material for water and the spreading performance of the material at an ice-water interface, wherein the material with strong spreading capability has good ice growth inhibition performance.

As a specific evaluation scheme of the step (c) of the present invention, the smaller the amount of an ice growth inhibition material required to cover a certain ice surface area, the better the spreading performance thereof, i.e., the spreading coefficient $S > 0$ is satisfied, wherein $S = \gamma_{I\text{-}W} - (\gamma_{IRIA\text{-}I} + \gamma_{IRIA\text{-}W})$, $\gamma_{I\text{-}W}$ being a constant, i.e., the ice-water interfacial energy $\gamma_{I\text{-}W}$ is greater than the sum of the interfacial energies of the material with ice and the material with water $\gamma_{IRIA\text{-}I} + \gamma_{IRIA\text{-}W}$ ($\gamma_{IRIA\text{-}I}$: interfacial energy of the material with ice; $\gamma_{IRIA\text{-}W}$: interfacial energy of the material with water).

In the present invention, the supercooling temperature refers to a temperature that is lower than the freezing point of water but at which water does not freeze or crystallize. At room temperature of 25 °C, the supercooling temperature is generally in the range of -0.01 to -0.5 °C, for example, -0.1 °C. The present invention also provides an ice adsorption experimental device, comprising a multilayer liquid storage cavity, a temperature-regulating rod and a temperature regulator, wherein the multilayer liquid storage cavity sequentially comprises an ice adsorption cavity, a bath cavity and a cooling liquid storage cavity from the inside to the outside, the temperature-regulating rod being arranged in the ice adsorption cavity, and the temperatures of the temperature-regulating rod and the liquid storage cavity being regulated by the temperature

regulator.

According to the ice adsorption experimental device of the present invention, the temperature-regulating rod is of a hollow structure made of a thermally conductive material, and the hollow structure of the temperature-regulating rod is provided with a liquid inlet and a liquid outlet; the temperature regulator is a fluid temperature regulator and is provided with a cooling liquid outflow end and a reflux end; two ends of the cooling liquid storage cavity is provided with a liquid inlet and a liquid outlet; the cooling liquid outflow end of the temperature regulator, the liquid inlet of the temperature-regulating rod, the liquid outlet of the temperature-regulating rod, the liquid inlet of a cooling liquid storage tank, the liquid outlet of the cooling liquid storage tank and the reflux end of the temperature regulator are sequentially linked via pipelines through which the cooling liquid flows.

According to the ice adsorption experimental device, the multilayer liquid storage cavity is provided with a cover.

When in use, the ice adsorption cavity is arranged to contain an aqueous solution of the ice growth inhibition material, and the bath cavity in the middle layer is arranged to contain a bath medium that is at a preset temperature, for example, a water bath, an ice bath or an oil bath; after the preset temperature of the cooling liquid is reached, the cooling liquid flows out through the temperature regulator and flows into the hollow structure of the temperature-regulating rod to regulate the temperature of the temperature-regulating rod, then flows out from the liquid outlet of the temperature-regulating rod and flows into the cooling liquid storage cavity in the outer layer to maintain the temperature of the bath medium at the preset level, and then flows through the liquid outlet of the cooling liquid storage tank and the reflux end of the temperature regulator and enters the temperature regulator to circulate.

The molecular design method and the screening method for the ice growth inhibition material of the present invention can be performed independently or in combination. In one embodiment, the present invention provides a full process method for designing and screening an ice growth inhibition material, comprising in sequence: the steps of designing the molecule according to the first aspect and the steps of screening the ice growth inhibition material according to the second aspect.

Specifically, the method comprises the following steps:

(1) constructing a library for structures of compound molecules, wherein the compound molecules comprise a hydrophilic group and an ice-philic group;

(2) simulating and evaluating the spreading performance of each of the compound molecules at an ice-water interface by adopting molecular dynamics (MD) simulation; and

(3) screening the ice growth inhibition molecules with desired affinities for ice and water according

to the step (2);

(4) synthesizing the screened ice growth inhibition molecules (ice growth inhibition material) with desired affinities for ice and water;

(5) determining the affinity of the ice growth inhibition material for water; and

(7) determining the spreading performance of the ice growth inhibition material at an ice-water interface.

According to the technical solutions of the present invention, the step (7) is followed by the step (c) of further evaluating the spreading performance, in which the affinity of the material for water and the spreading performance of the material at an ice-water interface are evaluated, wherein the material with strong spreading capability has good ice growth inhibition performance.

As a specific evaluation scheme of the step (c) of the present invention, the smaller the amount of an ice growth inhibition material required to cover a certain ice surface area, the better the spreading performance thereof, i.e., the spreading coefficient $S > 0$ is satisfied, wherein $S = \gamma_{I\text{-}W} - (\gamma_{IRIA\text{-}I} + \gamma_{IRIA\text{-}W})$, $\gamma_{I\text{-}W}$ being a constant, i.e., the ice-water interfacial energy $\gamma_{I\text{-}W}$ is greater than the sum of the interfacial energies of the material with ice and the material with water $\gamma_{IRIA\text{-}I} + \gamma_{IRIA\text{-}W}$ ($\gamma_{IRIA\text{-}I}$: interfacial energy of the material with ice; $\gamma_{IRIA\text{-}W}$: interfacial energy of the material with water).

According to the molecular design method and the screening method above, is the inventors of the invention found that the hydroxyl tacticity has an influence on the capability of a polyvinyl alcohol (PVA) to control the growth of ice crystals, and further found that a PVA with specific diad syndiotacticity has excellent capability of controlling the growth of ice crystals, wherein the PVA has a diad syndiotacticity r of 45%–60% and a molecular weight of 10–500 kDa; preferably, the PVA has a diad syndiotacticity r of 50%–55% and a molecular weight of 10–30 kDa.

In the present invention, various peptidic compounds, such as dipeptide, tripeptide, peptoid and glycopeptide compounds, are also designed and synthesized, and have excellent capability of controlling the growth of ice crystals.

The peptidic compounds are obtained by reacting ice-philic amino acids, such as threonine (L-Thr), glutamine (L-Gln) and aspartic acid (L-Asn) and the like, with other hydrophilic amino acids that may be selected from arginine, proline, alanine, and the like, or GDL or saccharides. The peptidic compounds consist of amino acids comprising an ice-philic group and amino acids comprising a hydrophilic group. In one embodiment, the amino acids composing the peptidic compounds are two or more types of amino acids, or one or more types of amino acids and GDL or saccharides.

In the present invention, it is also found that certain specific amino acids or polyamino acids have excellent capability of controlling the growth of ice crystals.

The amino acid is an amino acid comprising an ice-philic group and a hydrophilic group, and the polyamino acid is a homopolymer of an amino acid, for example, the polyamino acid is a homopolymer of the amino acid selected from arginine, threonine, proline, lysine, histidine, glutamic acid, aspartic acid, glycine and the like; the degree of polymerization is preferably 2–40, more preferably 2–20, for example, 6, 8, 15 and 20; for example, the polyamino acid is one of or a combination of two or more of poly-L-proline, poly-L-arginine.

Illustratively, the amino acid is selected from one or two of arginine, threonine, proline, lysine, histidine, glutamic acid, aspartic acid, glycine, and the like; and, for example, is a combination of arginine and threonine.

In a third aspect of the present invention, a cryopreservation solution is provided, comprising the ice growth inhibition material designed by the method according to the first aspect, or the ice growth inhibition material screened by the method according to the second aspect. In one embodiment, the ice growth inhibition material is one of or a combination of one or more of a PVA, an amino acid or a polyamino acid, and/or a peptidic compound; the cryopreservation solution also comprises a polyol, a water-soluble saccharide (or a derivative thereof such as water-soluble cellulose) and a buffer.

In one embodiment, the cryopreservation solution comprises a peptidic compound, and specifically comprises, per 100 mL, 0.1–50 g of the peptidic compound, 0–6.0 g of a PVA, 0–9.0 g of a polyamino acid, 0–15 mL of DMSO, 5–45 mL of a polyol, a water-soluble saccharide at 0.1–1.0 $mol \cdot L^{-1}$, 0–30 mL of serum, and the balance of a buffer.

In one embodiment, the cryopreservation solution comprises a PVA, and specifically comprises, per 100 mL, 0.01–6.0 g of a PVA, 0–50 g of the peptidic compound, 0–9.0 g of a polyamino acid, 0–15 mL of DMSO, 5–45 mL of a polyol, a water-soluble saccharide at 0.1–1.0 $mol \cdot L^{-1}$, 0–30 mL of serum, and the balance of a buffer.

In one embodiment, the cryopreservation solution comprises an amino acid or a polyamino acid, and specifically comprises, per 100 mL, 0.1–50 g of the amino acid or the polyamino acid, 0–6.0 g of a PVA, 0–15 mL of DMSO, 5–45 mL of a polyol, a water-soluble saccharide at 0.1–1.0 $mol \cdot L^{-1}$, 0–30 mL of serum, and the balance of a buffer. According to the present invention, the content of the amino acid and/or the polyamino acid in the cryopreservation solution may be 0.5–50 g, preferably 1.0–35 g, per 100 mL. For example, the content of the amino acid may be 5.0-35 g, preferably 15-25 g, in the presence of the amino acid; the content of the polyamino acid may be 0.5-9.0 g, preferably 1.0-5.0 g, in the presence of the polyamino acid.

According to the present invention, the polyol may be a polyol having 2–5 carbon atoms, preferably a diol having 2–3 carbon atoms, and/or a triol, such as any one of ethylene glycol, propylene glycol and glycerol; ethylene glycol is preferred.

According to the present invention, the water-soluble saccharide may be at least one of a non-reducing disaccharide, a water-soluble polysaccharide, a water-soluble cellulose and a glycoside, and, for example, is selected from sucrose, trehalose, hydroxypropyl methylcellulose and polysucrose; sucrose is preferred. The water-soluble saccharide can protect cell membranes and prevent cell sedimentation.

According to the present invention, the buffer may be selected from at least one of DPBS and hepes-buffered HTF buffer, or other cell culture buffer.

According to the present invention, the serum can comprise human serum albumin or a substitute thereof, such as sodium dodecyl sulfate, for a human-derived cryopreservation object, and can comprise fetal bovine serum or bovine serum albumin for a non-human-derived cryopreservation object.

According to the present invention, the content of the DMSO in the cryopreservation solution is 0–10 mL, preferably 1.0–7.5 mL, for example, 1.5–5 mL, per 100 mL; as another embodiment of the present invention, the content of the DMSO in the cryopreservation solution is 0 per 100mL.

According to the present invention, the content of the serum in the cryopreservation solution is 0.1–30 mL, for example, 5.0–20 mL, and 10–15 mL, per 100 mL; as another embodiment of the present invention, the content of the serum in the cryopreservation solution is 0 per 100 mL.

According to the present invention, the content of the water-soluble saccharide in the cryopreservation solution is 0.1–1.0 $mol \cdot L^{-1}$, for example, 0.1–0.8 $mol \cdot L^{-1}$, and 0.2–0.6 $mol \cdot L^{-1}$, per 100 mL; specifically, for example, 0.25 $mol \cdot L^{-1}$, 0.5 $mol \cdot L^{-1}$, and 1.0 $mol \cdot L^{-1}$.

According to the present invention, the content of the polyol in the cryopreservation solution is 5.0–40 mL, for example, 6.0–20 mL, and 9–15 mL, per 100 mL.

According to the present invention, the PVA may be selected from a PVA having a degree of hydrolysis of greater than 80%, for example, 80%–99%, 82%–87%, 87%–89%, 89%–99%, and 98%–99%.

As one embodiment of the present invention, the cryopreservation solution comprises the following components per 100 mL: 0.5–50 g of an amino acid, 5.0–45 mL of a polyol, 0–10 mL of DMSO, 0.1–30 mL of serum, a water-soluble saccharide at 0.1–1.0 $mol \cdot L^{-1}$, and the balance of a buffer. Preferably, the cryopreservation solution comprises the following components per 100 mL: 2.0–20 g of L-Arg, 1.0–10 g of L-Thr, 5.0–15 mL of ethylene glycol, 5.0–10 mL of DMSO, 5.0–20 mL of serum, sucrose at 0.1–1.0 $mol \cdot L^{-1}$, and the balance of DPBS.

As one embodiment of the present invention, the cryopreservation solution comprises the following components per 100 mL in volume: 0.5–9.0 g of a polyamino acid, 5.0–45 mL of a polyol, 0–10 mL of DMSO, 5.0–20 mL of serum, a water-soluble saccharide at 0.1–1.0 $mol \cdot L^{-1}$, and the balance of a buffer. Preferably, the cryopreservation solution comprises the following

components per 100 mL in volume: 1.0–8.0 g of poly-L-proline or poly-L-arginine, 5–45 mL of ethylene glycol, 0.1–10 mL of DMSO, 5.0–20 mL of serum, sucrose at 0.1–1.0 mol·L^{-1}, and the balance of DPBS.

As one embodiment of the present invention, the cryopreservation solution comprises the following components per 100 mL in velome: 0.01–6.0 g of a PVA, 5.0–45 mL of a polyol, 0.1–30 mL of a serum, a water-soluble saccharide at 0.1–1.0 mol·L^{-1}, and the balance of a buffer.

Preferably, the cryopreservation solution comprises the following components per 100 mL: 0.01–6.0 g of a PVA, 5.0–30 mL of ethylene glycol, 10–20 mL of serum, sucrose at 0.1–0.6 mol L^{-1}, and the balance of DPBS.

As one embodiment of the present invention, the cryopreservation solution comprises the following components per 100 mL: 1.0–5.0 g of a PVA, 5.0–20 mL of a polyol, 0.1–10 mL of DMSO, 0.1–20 mL of serum, a water-soluble saccharide at 0.2–0.8 mol·L^{-1}, and the balance of a buffer. Preferably, the cryopreservation solution comprises the following components per 100 mL: 1.0–4.0 g of a PVA, 5.0–15 mL of ethylene glycol, 4–10 mL of DMSO, 10–20 mL of serum, sucrose at 0.2–0.6 mol·L^{-1}, and the balance of DPBS.

As one embodiment of the present invention, the cryopreservation solution comprises the following components per 100 mL in velome: 0.1–6.0 g of a PVA, 10–45 mL of a polyol, a water-soluble saccharide at 0.2–1.0 mol·L^{-1}, and the balance of a buffer. Preferably, the cryopreservation solution comprises the following components per 100 mL in velome: 1.0–5.0 g of a PVA, 5.0–20 mL of ethylene glycol, sucrose at 0.2–0.6 mol·L^{-1}, and the balance of DPBS.

As one embodiment of the present invention, the cryopreservation solution comprises the following components per 100 mL in velome: 0.5–9.0 g of a polyamino acid, 5.0–45 mL of a polyol, 0.1–6 g of a PVA, 0–20 mL of serum, a water-soluble saccharide at 0.1–1.0 mol·L^{-1}, and the balance of a buffer. Preferably, the cryopreservation solution comprises the following components per 100 mL in velome: 1.0–8.0 g of poly-L-proline or poly-L-arginine, 5–45 mL of ethylene glycol, 0.1–6 g of a PVA, 5.0–20 mL of serum, sucrose at 0.1–1.0 mol·L^{-1}, and the balance of DPBS.

The present invention further provides a method for preparing the cryopreservation solution, comprising the following steps:

(1) weighting out any one or more of the amino acids or the polyamino acid, the PVA and the peptidic compound, separately dissolving in a portion of a buffer, and adjusting pH to form a solution;

(2) dissolving a water-soluble saccharide in the other portion of the buffer, and adding other components except serum after the water-soluble saccharide is completely dissolved to prepare a solution; and

(3) cooling the solutions of the step (1) and the step (2) to room temperature before mixing, adjusting the pH, and making up to a predetermined volume with the buffer to give the cryopreservation solution.

According to the preparation method of the cryopreservation solution of the invention, when the cryopreservation solution comprises serum, the serum is added when the cryopreservation solution is used.

According to the preparation method disclosed herein, the PVA is dissolved by heating in a warm bath and stirring, and for example, the heating is performed in a water bath or an oil bath; for example, the temperature of the water bath is 65–85 °C, or 70–80 °C; the stirring is mechanical stirring such as magnetic stirring.

According to the preparation method disclosed herein, the dissolution of the water-soluble saccharide is ultrasonic-assisted dissolution.

The cryopreservation solution disclosed herein can be used in combination with a freezing equilibration solution. In one embodiment, the present invention provides a freezing equilibration solution comprising, per 100 mL, 5.0–45 mL of polyol and the balance of a buffer.

The freezing equilibration solution disclosed herein further optionally comprises 0–15 mL of DMSO, 0–30 mL of serum, and/or 0–5.0 g of a PVA.

According to the freezing equilibration solution disclosed herein, the content of the polyol is 6.0–28 mL, for example, 7.0–20 mL, or 10–15 mL.

According to the freezing equilibration solution disclosed herein, the content of the DMSO is 0.1–15 mL, for example, 1.0–10 mL, or 5.0–7.5 mL; as one embodiment of the present invention, the content of the DMSO is 0.

According to the freezing equilibration solution disclosed herein, the content of the serum is 0.1–30 mL, for example, 5.0–20 mL, or 10–15 mL; as one embodiment of the present invention, the content of the serum is 0.

According to the freezing equilibration solution disclosed herein, the content of the PVA is 0.1–5.0 g, for example, 0.1 g, 0.5 g, 1.0 g, or 2.0 g; as one embodiment of the present invention, the content of the PVA is 0.

In the freezing equilibration solution disclosed herein, the polyol, serum, and buffer may be selected from the same types as those in the cryopreservation solution. In one embodiment, when the cryopreservation solution does not comprise serum, the freezing equilibration solution is added with a PVA.

As one embodiment of the present invention, the freezing equilibration solution comprises, per 100 mL, 5.0–7.5 mL of a polyol, 5.0–7.5 mL of DMSO, 10–20 mL of serum and the balance of a buffer.

As one embodiment of the present invention, the freezing equilibration solution comprises, per 100 mL, 7.5–15 mL of a polyol, 10–20 mL of serum and the balance of a buffer.

As one embodiment of the present application, the freezing equilibration solution comprises, per 100 mL, 1.0–5.0 g of a PVA, 7.5–15 mL of a polyol and the balance of a buffer.

The present invention further provides a method for preparing the freezing equilibration solution, comprising dissolving each component in a buffer, storing serum separately and adding the serum when the freezing equilibration solution is used.

A cryopreservation reagent comprises the above-mentioned freezing equilibration solution and the above-mentioned cryopreservation solution, wherein the freezing equilibrium solution and the cryopreservation solution are independently present.

According to the cryopreservation reagent disclosed herein, when the cryopreservation solution does not comprise serum, the freezing equilibration solution is added with a PVA.

Specifically, when the content of the DMSO in the cryopreservation solution is 0, the freezing equilibration solution comprises, per 100 mL, 0–5.0 g of a PVA, 7.5–15 mL of a polyol, 10–20 mL of serum and the balance of a buffer; when the contents of the DMSO and the serum in the cryopreservation solution are both 0, the freezing equilibration solution comprises, per 100 mL, 1.0–5.0 g of a PVA, 7.5–15 mL of a polyol and the balance of a buffer.

The cryopreservation solution or the cryopreservation equilibration solution disclosed herein or a combination thereof can be used for cryopreservation of various cells, tissues and organs. Various types of cells include, but are not limited to, germ cells such as oocytes and sperms, and various stem cells such as umbilical cord mesenchymal stem cells; various types of tissues include, but are not limited to, ovarian tissues, embryonic tissues and fertilized eggs; various types of organs include, but are not limited to, ovary or other mammalian organs.

Further, the present invention provides use of the above-mentioned cryopreservation solution or the cryopreservation equilibration solution or a combination thereof for cryopreservation of cells, tissues and organs. In one embodiment, the above-mentioned cryopreservation solution or cryopreservation equilibration solution or a combination of thereof is used for cryopreservation of oocytes; in one embodiment, the cryopreservation solution or cryopreservation equilibration solution or a combination thereof is used for cryopreservation of embryos; in one embodiment, the cryopreservation solution or cryopreservation equilibration solution or a combination thereof is used for cryopreservation of ovarian tissues or ovarian organs; in one embodiment, the cryopreservation solution or cryopreservation equilibration solution or a combination thereof is used for cryopreservation of stem cells.

The present invention further provides a method for freezing and thawing cells or embryos, comprising:

(1) placing the cells or embryos into the cryopreservation solution disclosed herein to prepare a cell suspension, and freezing; and

(2) placing the frozen cells or embryos into a thawing solution for thawing.

According to the method for freezing and thawing disclosed herein, the cells or the embryos are firstly placed into the equilibration solution for equilibration before being placed into the cryopreservation solution.

The present invention further provides a method of cryopreservation of stem cells, in which the microdroplet method is employed. For example, the method of cryopreservation of stem cells comprises the following steps: adding a cryopreservation solution into stem cells, pipetting to disperse the stem cells to prepare a stem cell suspension, and placing the stem cell suspension on a freezing slide and cryopreserving it in liquid nitrogen (-196 °C).

According to an embodiment of the present invention, the thawing of the cryopreserved stem cells comprises placing the freezing slide with the stem cells in an a-MEM medium and thawing the cells at 37 °C.

According to an embodiment of the present invention, the stem cells are various stem cells that are known in the art and capable of differentiating, such as totipotent, pluripotent or unipotent stem cells, including but not limited to embryonic stem cells, various types of mesenchymal stem cells (e.g., umbilical cord mesenchymal stem cells, adipose mesenchymal stem cells and bone marrow mesenchymal stem cells), hematopoietic stem cells, and the like.

The present invention further provides a method of cryopreservation of organs and/or tissues, comprising: placing an organ and/or a tissue into a freezing equilibration solution for equilibration, then placing the organ and/or the tissue into a cryopreservation solution, further placing the organ and/or the tissue on a freezing slide, and cryopreserving it in liquid nitrogen.

In one embodiment, the organ and/or the tissue is an ovarian tissue or an ovarian organ, which may be a slice of the ovarian tissue or a complete ovarian tissue.

In the present invention, “cryopreservation” and “cryogenic preservation” have the same meaning and are used interchangeably, and refer to preservation of a substance, or a cell, a tissue, or an organ at a low temperature to retain the original physicochemical and/or biological activity, and physiological and biochemical functions thereof.

In the present invention, the term “ice growth inhibition molecule” or “ice growth inhibition material” has the same meaning and refers to a compound capable of inhibiting the growth of ice crystals in an aqueous solution. In one embodiment, the ice growth inhibition molecule has good spreading performance at an ice-water interface and can reduce the grain size of ice crystals, or the ice growth inhibition molecule has no thermal hysteresis or has sufficiently small thermal hysteresis to significantly reduce ice crystal formation in an aqueous solution.

Beneficial Effects

1. In the present invention, a mechanism of controlling the growth of ice crystals in an ice-water mixed phase by an ice growth inhibition molecule is found for the first time. The ice growth inhibition material is required to have good affinities for both ice and water. The affinity of the ice growth inhibition molecules for ice can ensure that the ice growth inhibition molecules are well adsorbed on the surface of the ice; the affinity of the molecules for water can ensure that the molecules better spread on an ice-water interface to cover the maximum ice surface area with as less amount of material as possible. Based on the ice growth inhibition mechanism, an idea of designing an ice growth inhibition molecule with affinities for both ice and water is proposed, which provides a new method for synthesizing an ice growth inhibition material.

2. In the present invention, MD simulation is introduced into the design of the molecular structure of an ice growth inhibition material for the first time, through which the affinities of the designed ice growth inhibition molecule for ice and water are evaluated, the ice growth inhibition performance of the ice growth inhibition material is predicted and thus optimization of the structure can be achieved.

3. In the present invention, the combination of the ice growth inhibition mechanism and MD simulation well solves the limitation that the materials known in the art can be subjected to performance analysis and screening only by the experimental “trial and error” approach in the research and development process of an ice growth inhibition material, provides a new idea of the design of a molecular structure and can significantly promote the development and application of the ice control material.

4. The cryopreservation solution provided herein has the advantages of wide sources, good biocompatibility, low toxicity, high safety and a greatly reduced amount of DMSO used, and can achieve an equal or even higher cell survival rate than that of a commercial cryopreservation solution comprising DMSO of no less than 15% and commonly available in clinical practice at present when used even without the addition of DMSO. The cryopreservation solution disclosed herein has advantages of simple composition, readily available starting materials and low costs, and can be widely applied to cryopreservation of various cells and tissues, such as oocytes, embryos, stem cells, ovarian tissues and ovarian organs, to retain good biological activity.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1: a schematic diagram of the molecular structure of the ice growth inhibition material;

FIG. 2: aggregation states of an atactic PVA (a-PVA) and an isotactic PVA (i-PVA) at an ice-water interface by MD simulation;

FIG. 3: a proton nuclear magnetic resonance (NMR) spectrum of the a-PVA synthesized in Example 1;

FIG. 4: proton NMR spectra of the PBVE and the i-PVA synthesized in Example 1, wherein A is for the PBVE and B is for the i-PVA;

FIG. 5: GPC curves of the PBVE synthesized in Example 1;

FIG. 6: dispersion sizes of the a-PVA (A) and the i-PVA (B) in water at different concentrations in the DLS experiment;

FIG. 7: micrographs of ice crystal growth in solutions of the two PVAs, wherein A is for the a-PVA and B is for the i-PVA; C shows the relationship of the two PVAs between the mean largest grain size and the concentration relative to that of PBS;

FIG. 8: the topography of ice crystals modified by the a-PVA (FIGs. A and B) and i-PVA (FIGs. C and D) in purified water;

FIG. 9: molecular structure models of the two PVAs by MD simulation;

FIG. 10: contactable surface areas of the two PVA molecules with water molecules and ice-water molecules at an ice-water interface at 240 K by MD simulation, wherein the upper part of the figure shows 3 results of the a-PVA molecules, and the lower part of the figure shows 3 results of the i-PVA molecules;

FIG. 11: aggregation probabilities of the two PVAs in an aqueous solution by MD simulation and calculations;

FIG. 12: the number of the intermolecular hydrogen bonds formed between the two PVAs and water at 240 K in an aqueous solution, and the number of the intermolecular hydrogen bonds formed between the two PVAs and water molecules and ice-water molecules at 240 K at an ice-water interface by MD simulation and calculations;

FIG. 13: optical micrographs of the inhibition of the growth activity of ice crystals by GDL-L-Thr (compound of formula (6)) and a statistical diagram of grain size;

FIG. 14: the topography of an ice crystal modified by GDL-L-Thr in purified water;

FIG. 15: optical micrographs of the inhibition of the growth activity of ice crystals by GDL-L-Ser (compound of formula (7)) and a statistical diagram of grain size;

FIG. 16: optical micrographs of the inhibition of the growth activity of ice crystals by GDL-L-Val (compound of formula (8)) and a statistical diagram of grain size;

FIG. 17: optical micrographs of the inhibition of the growth activity of ice crystals by the TR short-chain peptide prepared in Example 3 and a statistical diagram of grain size;

FIG. 18: the topography of an ice crystal modified by the TR short-chain peptide prepared in Example 3 in purified water;

FIG. 19: inhibition results of the growth activity of ice crystals by the peptoids R-COOH, R-CH_3

and R-CH_2CH_3 in Example 8;

FIG. 20: the topographies of ice crystals modified by the peptoids R-COOH (A), R-CH_3 (B) and R-CH_2CH_3 (C) in Example 8 in purified water;

FIG. 21: a schematic diagram of an ice adsorption experiment and a device thereof;

FIG. 22: diagrams of the ice adsorption amount vs. the concentration for the two PVAs in Example 9;

FIG. 23: micrographs of ice crystal growth in DPBS solutions of the two PVAs, wherein A is for the a-PVA and B is for the i-PVA;

FIG. 24: a picture of a stained slice of a fresh (unfrozen) ovarian organ of a 3-day-old newborn mouse;

FIG. 25: a picture of a stained slice of the cryopreserved ovarian organ in Comparative Embodiment 8 after thawing;

FIG. 26: a picture of a stained slice of the cryopreserved ovarian organ in Application Embodiment 13 after thawing;

FIG. 27: a picture of a stained slice of the cryopreserved ovarian organ in Application Embodiment 14 after thawing;

FIG. 28: a picture of a stained slice of the cryopreserved ovarian organ in Application Embodiment 15 after thawing;

FIG. 29: a picture of a stained slice of fresh (unfrozen) ovarian tissue of a sexually mature mouse;

FIG. 30: a picture of a stained slice of the cryopreserved ovarian tissue in Comparative Embodiment 9 after thawing;

FIG. 31: a picture of a stained slice of the cryopreserved ovarian tissue in Application Embodiment 16 after thawing;

FIG. 32: a picture of a stained slice of the cryopreserved ovarian tissue in Application Embodiment 17 after thawing;

FIG. 33: a picture of a stained slice of the cryopreserved ovarian tissue in Application Embodiment 18 after thawing;

FIG. 34: a picture of a stained slice of the cryopreserved ovarian tissue in Application Embodiment 26 after thawing;

FIG. 35: a picture of a stained slice of the cryopreserved ovarian tissue in Application Embodiment 27 after thawing;

FIG. 36: a picture of a stained slice of the cryopreserved ovarian tissue in Application Embodiment 28 after thawing;

FIG. 37: a picture of a stained slice of the cryopreserved ovarian tissue in Application

Embodiment 29 after thawing;

FIG. 38: a picture of a stained slice of the cryopreserved ovarian tissue in Application Embodiment 30 after thawing;

FIG. 39: a picture of a stained slice of the cryopreserved ovarian tissue in Application Embodiment 31 after thawing;

FIG. 40: a picture of a stained slice of the cryopreserved ovarian tissue in Application Embodiment 37 after thawing; and

FIG. 41: a picture of a stained slice of the cryopreserved ovarian tissue in Application Embodiment 38 after thawing.

DETAILED DESCRIPTION

The preparation method of the present invention will be further illustrated in detail with reference to the following specific examples. It should be understood that the following examples are merely exemplary illustration and explanation of the present invention, and should not be construed as limiting the protection scope of the present invention. All techniques implemented based on the afore-mentioned contents of the present invention are encompassed within the protection scope of the present invention.

Unless otherwise stated, the experimental methods used in the following examples are conventional methods. Unless otherwise stated, the reagents, materials, and the like used in the following examples are commercially available.

A. Molecular design of ice growth inhibition material

The core molecule of the ice growth inhibition material disclosed herein can be designed to various groups having an affinity for water and groups having an affinity for ice, which are linked by covalent bonds or non-covalent bonds such as ionic bonds.

The molecular design method for the ice growth inhibition material disclosed herein comprises the following steps:

(1) constructing a library for structures of compound molecules, wherein the compound molecules comprise a hydrophilic group and an ice-philic group;

(2) simulating and evaluating the spreading performance of each of the compound molecules at an ice-water interface by adopting molecular dynamics (MD) simulation; and

(3) screening the ice growth inhibition molecule with desired affinities for ice and water.

According to the present invention, the main chain of the ice growth inhibition molecule is a carbon chain or a peptide chain.

According to the present invention, the MD simulation of the step (2) can be performed by GROMACS, AMBER, CHARMM, NAMD, or LAMMPS.

According to the present invention, in the MD simulation of the step (2), a model of a water molecule may be selected from models of TIP3P, TIP4P, TIP4P/2005, SPC, TIP3P, TIP5P, and SPC/E, preferably TIP4P/2005 model of a water molecule.

According to the present invention, in the MD simulation of the step (2), a force field parameter is provided by one of GROMOS, ESFF, MM force field, AMBER, CHARMM, COMPASS, UFF, CVFF and other force fields.

According to the present invention, in the MD simulation of the step (2), simulation and calculation are performed on interactions between ice growth inhibition molecules, interactions between ice growth inhibition molecules and water molecules, and interactions between ice growth inhibition molecules and ice-water molecules. The interactions include the formation of a hydrogen bond, a Van der Waals interaction, an electrostatic interaction, a hydrophobic interaction, a π-π interaction and the like.

According to the present invention, in the MD simulation of the step (2), when the simulated and calculated molecules interact, the temperature and pressure are adjusted. In one embodiment of the present invention, a V-rescale (modified Berendsen) temperature-regulator and a pressure-regulator are used to regulate the temperature and pressure.

According to the present invention, in the MD simulation of the step (2), the molecular configuration of the compound molecule is maintained by selecting a potential energy parameter. Preferably, the potential energy parameter is selected, so that the molecular configuration of the compound molecule is maintained at a higher temperature.

According to the present invention, in the step (2), periodic boundary conditions are adopted for x-direction, y-direction and z-direction when an aqueous solution system is simulated; periodic boundary conditions are adopted for x-direction and y-direction when an ice-water mixed system is simulated.

According to the present invention, in the MD simulation of the step (2), a cubic or octahedral box of water is selected, and a cubic box of water with dimensions of $3.9 \times 3.6 \times 1.0$ nm^3 is preferred.

As a specific embodiment of the present invention, during the process of molecular dynamics calculations of the MD simulation, the V-rescale (modified Berendsen) temperature-regulator and the pressure-regulator regulate the temperature and pressure.

In the MD simulation and calculations, the main criterion for determining the existence of a hydrogen bond is the energy criteria or the geometric criteria, preferably, the geometrical criteria; when the distance (pitch) between oxygen atoms is less than 0.35 nm and the angle HO...H is less than 30 degrees, a hydrogen bond between two hydroxyl groups or between a hydroxyl group and a water molecule is formed.

As a specific embodiment of the present invention, the ice growth inhibition material may be a compound that has a carbon chain as the main chain and is substituted with an ice-philic group and a hydrophilic group; the ice growth inhibition material may comprise a group that is both hydrophilic and ice-philic, such as hydroxyl and amino groups, and may further comprise an ice-philic group and a hydrophilic group separately. For example, the molecular structure of the ice growth inhibition material is designed to have a repeating unit -[CH_2-CHOH]-.

In an embodiment of the present invention, the molecule of the ice growth inhibition material is a PVA. The PVA is selected from one of or a combination of two or more of an isotactic PVA, a syndiotactic PVA and an atactic PVA. For example, the PVA has a diad syndiotacticity of 15%–65%, specifically, for example, 40%–60% and 53%–55%. Atactic PVA is preferred, for example, the PVA with a diad syndiotacticity of 45%–65%. The PVA may be selected from a PVA having a molecular weight of 10–500 kDa or higher, such as 10–30 kDa, 30–50 kDa, 80–90 kDa, and 200–500 kDa. The PVA may be selected from a PVA having a degree of hydrolysis of greater than 80%, for example, 80%–99%, 82%–87%, 87%–89%, 89%–99%, and 98%–99%.

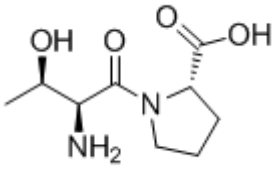
In an embodiment of the present invention, the molecule of the ice growth inhibition material is a peptidic compound. The peptidic compounds are obtained by reacting ice-philic amino acids, such as threonine (L-Thr), glutamine (L-Gln) and aspartic acid (L-Asn), with other hydrophilic amino acids that may be selected from arginine, proline, alanine, and the like, or GDL or saccharides.

The peptidic compound consists of no less than two amino acid units, such as: 2–8 amino acid units, specifically, 2–5, such as 2, 3, 4, 5 and 6 amino acid units; each amino acid unit is different. In the peptidic compound, the molar ratio of the ice-philic amino acid such as threonine to other hydrophilic amino acids is (0.1–3):1, preferably (0.5–2):1. The arrangement of the ice-philic amino acid and other hydrophilic amino acids in the peptidic compound is not particularly limited, and may be linked by using the amino acid linking groups or chemical bonds known in the art. For example, the ice-philic amino acid and the hydrophilic amino acid may be alternately arranged, or multiple ice-philic amino acids or hydrophilic amino acids are linked to form a fragment of the ice-philic amino acid or the hydrophilic amino acid, which is then linked to the hydrophilic amino acid (or a fragment) or the ice-philic amino acid (or a fragment), respectively.

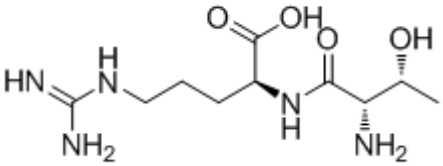
In an embodiment of the present invention, the peptidic compound is at least one of L-Thr-L-Arg (TR), L-Thr-L-Pro (TP), L-Arg-L-Thr (RT), L-Pro-L-Thr (PT), L-Thr-L-Arg-L-Thr (TRT), L-Thr-L-Pro-L-Thr (TPT), L-Ala-L-Ala-L-Thr (AAT) and L-Thr-L-Cys-L-Thr (TCT).

In another embodiment, the peptidic compound is a GDL-L-amino acid, such as GDL-L-Thr, GDL-L-Ser or GDL-L-Val.

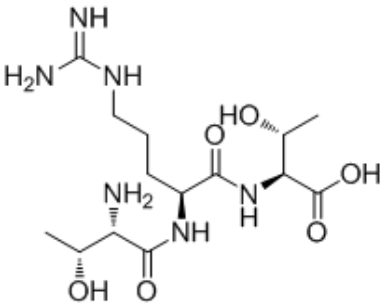
In yet another embodiment, the peptidic compound has any one of the structures of formula (1) to formula (8):



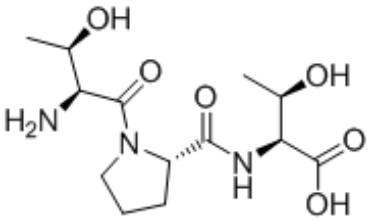
formula (1)



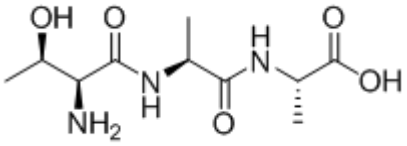
formula (2)



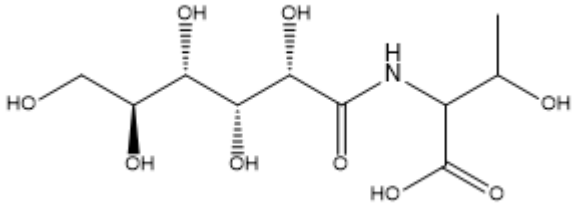
formula (3)



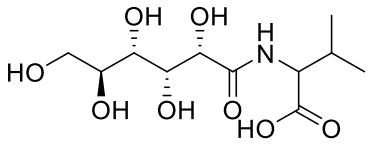
formula (4)



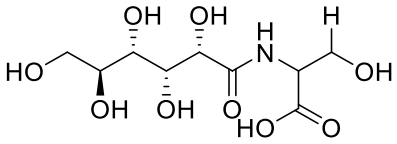
formula (5)



formula (6)



formula (7)



formula (8).

The above-mentioned peptidic compounds can be synthesized using a polypeptide synthesis method known in the art, such as a solid-phase synthesis method.

The preparation method disclosed herein comprises the following steps: resin swelling, covalently linking an amino acid whose the amino group is protected to the swollen resin, deprotecting, adding another amino acid whose the amino group is protected for condensation reaction, deprotecting, cleavaging and purifying.

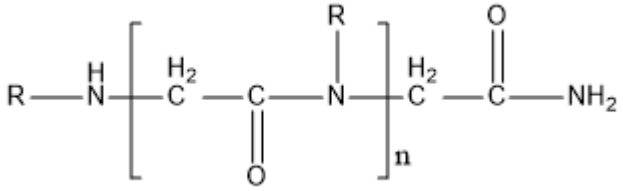
The glycopeptide derivative can be prepared by using the known method for reacting an amino acid with a saccharide. For example, the glycopeptide derivative can be prepared by reacting glucono delta-lactone or other saccharides with an amino acid in an organic solvent, or by using a solid-phase synthesis method. Glucono delta-lactone (GDL) is dissolved in an organic solvent, an amino acid and an alkaline catalyst are added to an organic solvent, and then the resulting mixture is added to the GDL solution to react at 55–60 °C after the amino acid and the alkaline catalyst are completely dissolved, after the reaction is finished, a white precipitate is filtered out, and the filtrate is evaporated to dryness to give a crude product.

According to the preparation method disclosed herein, the organic solvent may be selected from methanol, ethanol and the like.

In one embodiment, the glycopeptide derivative is prepared by using a solid-phase synthesis method comprising: resin swelling, covalently linking an amino acid where the amino group is protected to the swollen resin, deprotecting, adding a saccharide (such as ring-opened GDL) for condensation reaction, cleavaging, and purifying. GDL-L-Val and GDL-L-Ser were synthesized

with reference to the method for synthesizing GDL-L-Thr.

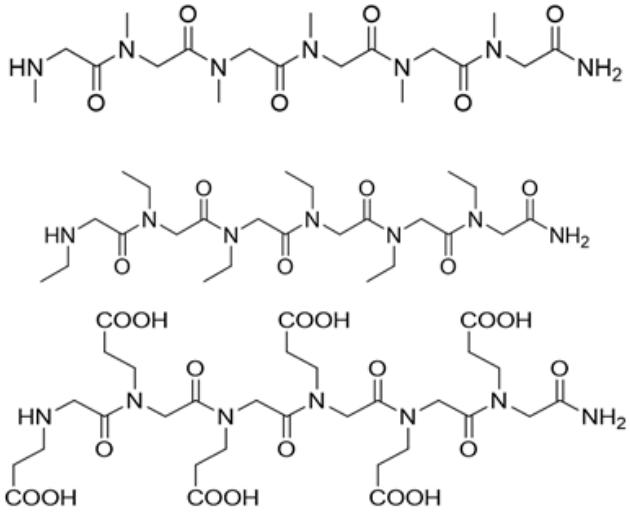
The present invention further provides a peptidic compound of formula (9):



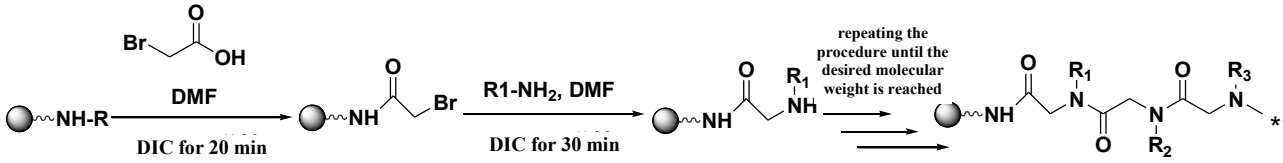
formula (9),

wherein R is selected from substituted or unsubstituted alkyl, and the substituent may be selected from -OH, -NH_2, -COOH, -$CONH_2$ and the like; for example, R is substituted or unsubstituted C_{1-6} alkyl, and preferably R is -CH_3, -CH_2CH_3, -CH_2CH_2 COOH; n is an integer no less than 1 and no more than 1000, and for example, may be an integer ranging from 1 to 100. In some embodiments of the present invention, n is an integer such as 2, 3, 4, 5, 6, 7, 8, 9 and 10.

As an embodiment of the present invention, the compound of formula (9) has a structure shown in any one of the following:



According to the present invention, the compound of formula (9) is prepared by using the following synthetic route:



In an embodiment of the present invention, the molecule of the ice growth inhibition material is an amino acid or a polyamino acid. The present invention further provides use of the above-mentioned molecule of the ice growth inhibition material, such as the PVA, the peptidic compound, the amino acid and the polyamino acid for controlling the growth of ice crystals in an

aqueous solution, and use of the peptidic compound for preparing a cryopreservation solution for cells or tissues.

The ice growth inhibition materials designed and prepared according to the present invention, such as the PVA, the peptidic compound, the amino acid and the polyamino acid, are used for preparing a cryopreservation solution for cryopreservation of cells, tissues, organs and the like.

[Example 1]

(1) Molecular structure design of compound:

Compound molecules having the repeating unit -[CH_2-CHOH]- were designed to give a library for molecular structures that includes molecular models of an atactic and isotactic PVA.

(2) MD simulation experiment

The differences in the affinities of the atactic PVA and the isotactic PVA for ice and water were predicted by MD simulation experiments.

a. MD simulation was performed by GROMACS 5.1, and the model of water was TIP4P/2005, which has a melting point of about 252.5 K. The interaction parameters of the PVA molecules were provided by the GROMOS54A7 force field, and the leapfrog integration algorithm with an integration step size of 2 fs was adopted. The electrostatic interaction was calculated by the PME method, and the cutoff radii of the coulomb potential and the L-J potential were both 1.0 nm. A V-rescale (modified Berendsen) temperature-regulator and a pressure-regulator were used to regulate the temperature and pressure. The time constant was set to 0.1 ps.

b. Molecular chains of compounds having 7 repeating units were simulated and selected for investigation. The topology files of the PVA molecules were generated through ATB, and in order to maintain the tacticities of the two PVA molecules, the dihedral angle potential functions of the carbon chains of the molecules were required to be adjusted correspondingly.

c. Periodic boundary conditions were adopted for x-direction, y-direction and z-direction when an aqueous solution system of the PVAs was simulated; periodic boundary conditions were adopted for x-direction and y-direction when an ice-water mixed system was simulated. All systems were simulated for 120 ns, and data from the last 60 ns were used for analysis.

The aqueous solution system of the molecules was first investigated. In a system having only one PVA chain, 1491 water molecules in total were used, the pressure was 1 atm, and the temperatures were 240 K, 250 K, 260 K, 270 K, 300 K, and 330 K.

In a system for investigating interactions of PVA molecules with ice, 6 PVA molecular chains were placed in a box of water with dimensions of $3.9 \times 3.6 \times 1.0$ nm^3, an ice block comprising 1100 water molecules was equilibrated at 240 K for 10 ns, and the ice block was placed under the box of water along the z-axis. The size of the mixed system in the z-direction was increased to 10 nm, and the ice-water mixed system was placed at the center of the box of water.

The topology files of the PVA molecules were generated through ATB, and the topology files were directly used. In order to maintain the tacticities of the two PVA molecules, the potential energy parameters were set to be 50 kcal/mol, so that the molecular configurations of the two PVA molecules can be maintained even at a higher temperature.

Molecular structure models of the two PVAs by MD simulation are shown in FIG. 9.

(3) Evaluation of simulation

The a-PVA can effectively generate hydrogen bonding with an ice surface and thus be adsorbed on the ice surface because the distance of three times the distance between adjacent OH matches the size of the ice crystal lattice. The i-PVA only changes the direction of the hydroxyl group without changing the distance between adjacent OH, so that the i-PVA and the a-PVA have similar ice adsorption ability. Meanwhile, according to the MD simulation results, the number of the intermolecular hydrogen bonds formed between the a-PVA and water molecules is more than that of the intermolecular hydrogen bonds formed between the i-PVA and water molecules, which indicates that the affinity of the a-PVA for water is stronger than that of the i-PVA. In addition, the states of 6 PVA molecular chains at an ice-water interface simulated by the MD simulation show that, the a-PVA tends to spread at an ice-water interface due to its good affinities for both ice and water while the i-PVA tends to aggregate at an ice-water interface due to its weaker affinity for water (FIG. 2).

Table 1

	a-PVA		i-PVA	
T / K	Intramolecular hydrogen bonding	Intermolecular hydrogen bonding	Intramolecular hydrogen bonding	Intermolecular hydrogen bonding
240	0.72(0.12)	6.76(0.43)	1.67(0.20)	5.92(0.42)
250	0.73(0.13)	6.87(0.32)	1.63(0.20)	5.95(0.36)
260	0.74(0.14)	6.83(0.35)	1.59(0.16)	6.04(0.35)
270	0.70(0.11)	6.87(0.30)	1.54(0.18)	6.13(0.34)
300	0.70(0.12)	6.74(0.35)	1.50(0.19)	6.09(0.28)
330	0.70(0.14)	6.54(0.33)	1.45(0.18)	6.05(0.31)

The MD simulation shows the contactable areas of the two PVAs with water molecules at an ice-water interface at 240 K, in which the contactable area of a-PVA is larger than that of the i-PVA, which further confirms that the spreading performance of the a-PVA at an ice-water interface is better than that of the i-PVA (see FIG. 10). The aggregation probability of the i-PVA in the aqueous solution calculated by MD is obviously higher than that of the a-PVA (FIG. 11); at 240K, the numbers of the hydrogen bonds formed between the two PVAs and ice-water molecules at an

ice-water interface is similar, but the number of the hydrogen bonds formed between the a-PVA and water at an ice-water interface and in the aqueous solution is obviously more than that of the i-PVA. Therefore, the a-PVA can better spread at an ice-water interface while the i-PVA aggregates (FIG. 12).

Therefore, multiple results of the MD simulation show that the a-PVA has better spreading performance at an ice-water interface due to a strong affinity of its molecular structure for water molecules, and thus has a better ice growth inhibition effect than the i-PVA.

(4) Synthesis of designed PVA molecules

(4.1) Preparation of atactic polyvinyl alcohol a-PVA: the molecular weight is about 13–23 kDa, and the diad syndiotacticity r is about 55%

Vinyl acetate (VAc, Sigma-Aldrich) from which the inhibitor had been removed was dissolved in 100 mL of a solvent (methanol) in a 250 mL round-bottom flask under argon atmosphere to give a 25%–45% solution of VAc. After being cooled to -5 °C, the reaction solution was carefully added dropwise with 80 mM of 2,2'-Azobis(2-methylpropionitrile) (Sigma-Aldrich). After being left to warm to room temperature, the above solution was stirred for 15 h, and the reaction solution was dissolved with 1 L of acetone and added dropwise to methanol to give a white precipitate. The above precipitate was washed with methanol, filtered and dried in an oven at 60 °C under vacuum for 6.0 h to give a white solid. The white solid was dissolved in a methanol solution (10 wt.%), and argon gas was introduced to remove oxygen from the solution. A 25% methanol solution of potassium hydroxide was added dropwise to the above solution and stirred for 4 h. After the stirring, the reaction solution was dissolved in a 2 M hydrochloric acid solution and added to a 2.0 M methanol solution of ammonia for precipitatation to give an atactic polyvinyl alcohol (a-PVA). The proton NMR spectrum (FIG. 3) shows that the compound obtained is a fully hydrolyzed a-PVA.

(4.2) Preparation of isotactic polyvinyl alcohol i-PVA: the molecular weight is about 14–26 kDa, and the isotacticity m is about 84%

a. Preparation of poly-*tert*-butyl vinyl ether (PBVE). *Tert*-butyl vinyl ether (*t*-BVE, Sigma-Aldrich) was dissolved in 100 mL of dry toluene in a 250 mL round-bottom flask under argon atmosphere to give a 2.5% toluene solution of *t*-BVE. After being cooled to -78 °C, the above solution was carefully added dropwise with 0.2 mM boron trifluoride diethyl ether ($BF_3 \cdot OEt_2$, Sigma-Aldrich), and supplemented with 0.2 mM $BF_3 \cdot OEt_2$ 2.0 h later. After the above solution was stirred at -78 °C for 3.0 h, the reaction was stopped with a small amount of methanol. The reaction solution was added dropwise to 2.0 L of methanol with rapid stirring to give a light yellow precipitate. The precipitate was washed with methanol, filtered and dried in an oven at 60 °C under vacuum for 6.0 h to give a light yellow solid powder, which was PBVE as shown in the

proton NMR spectrum (FIG. 4A). The molecular weight of the synthesized PBVE was regulated by adjusting the concentrations of boron trifluoride diethyl ether and *tert*-butyl vinyl ether. PBVE with different molecular weights was successfully synthesized as shown by the gel permeation chromatography (GPC) chromatogram (obtained using tetrahydrofuran (THF) system, flow rate of 1 $mL \cdot min^{-1}$) (FIG. 5).

b. Preparation of dry hydrogen bromide gas (HBr); in a 100 mL two-neck flask, 5.0–30 mL of phosphorus tribromide (PBr_3, Aladdin) was added dropwise to 10 mL of 48% aqueous solution of hydrogen bromide (HBr, Alfa Aesar). The resulting gas was allowed to sequentially pass through tetrachloromethane (CCl_4), red phosphorus (P, Alfa Aesar) and calcium chloride ($CaCl_2$) to give a dry HBr gas.

c. Preparation of isotactic polyvinyl alcohol (isotactic-PVA, i-PVA). 0.5 g of PBVE was dissolved in 15 mL of dry toluene under argon atmosphere, and dry argon was continuously introduced to remove oxygen from the resulting solution. The dry HBr gas prepared in the step b was allowed to pass into the above oxygen-free toluene solution of PBVE at 0 °C. After about 5.0 min, a light yellow precipitate formed, and the introduction of dry HBr gas was continued until no precipitate formed. The above reaction solution was poured into 200 mL of methanol solution of ammonia (2.0 M).The resulting precipitate was washed with methanol, filtered, and dried in an oven at 60 °C under vacuum for 6.0 h to give a light yellow solid powder. The proton NMR spectrum (FIG. 4B) shows that the hydrolysis of PBVE was complete to give a solid i-PVA.

(5) Verification of ice growth inhibition effect of synthesized PVA

(5.1) Dynamic light scattering (DLS) experiment

The grain size distributions of the two PVAs (a-PVA: the molecular weight of about 13–23 kDa, the diad syndiotacticity r of about 55% (Sigma-Aldrich); i-PVA: the molecular weight of about 14–26 kDa, the isotacticity m of about 84%) in an aqueous solution at 25 °C were measured by a DLS experiment, and an experimental instrument was a Nano ZS (Malvern Instruments) with a thermostatic chamber and a 4 mW He-Ne laser (λ = 632.8 nm), wherein the scattering angle is 173°. Firstly, aqueous solutions of the a-PVA and the i-PVA at the concentrations of 1.0 $mg \cdot mL^{-1}$, 4.0 $mg \cdot mL^{-1}$, 10 $mg \cdot mL^{-1}$ and 20 $mg \cdot mL^{-1}$ were prepared; about 1.0 mL of each the PVA solution was added into a 12 mm disposable polystyrene cuvette for measurement.

The results of the DLS experiment show that when being at the same concentration, the a-PVA has a much smaller dispersion size in an aqueous solution than the i-PVA (FIG. 6). That is, compared to the a-PVA, the i-PVA tends to exist in an aggregated state in an aqueous solution. This is consistent with the results that in the MD simulation, the number of the intramolecular hydrogen bonds of the a-PVA is less than that of the i-PVA, and the number of the intermolecular

hydrogen bonds formed between the a-PVA and water molecules is more than that formed between the i-PVA and water molecules.

(5.2) Assay for ice recrystallization inhibition (IRI) activity

The IRI activity was assessed using "splat-freezing method", wherein a sample was dissolved and dispersed into a DPBS solution, and 10–30 μL of the resulting solution was added dropwise onto the surface of a clean silicon disk pre-cooled at -60 °C at a height of no less than 1.0 m; the solution was slowly heated to -6 °C at a speed of 10 °C·min^{-1} by using a hot-cold stage, and was annealed for 30 min at this temperature; the sizes of ice crystals were observed and recorded by using a polarizing microscope and a high-speed camera. The hot-cold stage was sealed to ensure that the internal humidity was about 50%. The procedure was repeated at least three times for each sample, and the sizes of ice crystals were counted using a Nano Measurer 1.2, with the error of the result being the standard deviation.

(5.3) Ice topography (DIS) observation and thermal hysteresis (TH) measurement

DIS observation and TH measurement were performed by using a nanoliter osmometer. A capillary was first melt with the outer flame of a alcohol burner, and simultaneously stretched to produce a capillary with a very fine pore size, and the capillary was then linked to a microsyringe; an immersion oil with higher viscosity was injected into a disk with micron-sized holes, and an aqueous solution in which the material was dissolved was injected into the microholes by using a microsyringe; the droplet was quickly frozen by regulating the temperature, and then slowly heated to give a single crystal ice, the single crystal ice was slowly cooled at the precision of 0.01 °C, and the DIS observation and TH test were performed by using a microscope provided with a high-speed camera.

The ability of the a-PVA (M_w 13–23 kD) to inhibit the growth of ice crystals is far better than that of the i-PVA (M_w 14–26 kD) with the corresponding molecular weight (FIG. 7). As can be seen in FIG. 7A, the grain size of the a-PVA is obviously smaller than that of the i-PVA at the same concentration; as can be seen in FIG. 7B, the mean largest grain size (MLGS) of the ice crystal of the a-PVA relative to that of DPBS reaches a minimum after 2.0 mg·mL^{-1}, the minimum being about 20% of the MLGS of the ice crystal of DPBS; the MLGS of the i-PVA of different molecular weights relative to that of DPBS increases with the increasing concentration and reaches a minimum at 10 mg mL^{-1}, the minimum being only about 50% of the MLGS of the ice crystal of DPBS, and the MLGS does not decrease but increase slightly with the concentration continuing to increase to 20 mg mL^{-1}. The i-PVA (M_w 14–26 kD) with the degree of polymerization of more than 333 was difficult to dissolve at the concentration of more than 30 mg mL^{-1}. Therefore, due to the limitation of the solubility of the i-PVA, the IRI activity of the i-PVA was optimally 50% of the MLGS of DPBS when the concentration was 10 mg mL^{-1}, and

the IRI activity of the a-PVA is optimally 20% of the MLGS of DPBS when the concentration was 2.0 mg mL^{-1}. This is consistent with the results that the a-PVA spreads more easily at an ice-water interface than the i-PVA in the MD simulation, which achieves the effect that the a-PVA can better inhibit the growth of ice crystals at a lower dosage than the i-PVA.

As can be seen from the results of the MD simulation and the actual verification experiment, the results are good in consistency. The ice growth inhibition performance of the ice growth inhibition material can be accurately predicted by MD simulation, and the molecular design of the ice growth inhibition material can be effectively achieved.

Compounds of formula (1) to formula (9) were designed by the same molecular design method, synthesized and studied for their ice growth inhibition effects.

[Example 2] Synthesis of compound of formula (1)

(1) 2-chlorotrityl chloride resin was placed into a reaction tube, and added with DCM (20 $mL \cdot g^{-1}$). The resulting mixture was shaken for 30 min. With the use of a sand-core funnel by suction, the solvent was removed. The residue was added with a three-fold molar excess of Fmoc-L-Pro-OH and an eight-fold molar excess of DIEA, and finally added with DMF to dissolve. The resulting mixture was shaken for 30 min. Methanol was used for end-capping for 30 min.

(2) The solvent DMF was removed. 20% piperidine/DMF solution (10 $mL \cdot g^{-1}$) was added, and the solvent was removed after 5 min; 20% piperidine/DMF solution (10 $mL \cdot g^{-1}$) was added again, and the piperidine solution was removed after 15 min. A small amount of resin was taken and washed with ethanol three times, added with a ninhydrin reagent, and heated at 105–110 °C for 5 min. The color turned dark blue, which suggested a positive reaction.

(3) After the product obtained by the above reaction was sequentially washed with DMF (15 $mL \cdot g^{-1}$, twice), methanol (15 $mL \cdot g^{-1}$, twice) and DMF (15 $mL \cdot g^{-1}$, twice), a two-fold excess of Fmoc-L-Thr(tBu)-OH that was dissolved in as small an amount of DMF as possible was added to a reaction tube; a two-fold excess of HBTU was added. Immediately thereafter, an eight-fold excess of DIEA was added and reacted for 30 min.

(4) After the solution was removed by suction, a small amount of resin was taken and washed with ethanol three times, added with a ninhydrin reagent, and heated at 105–110 °C for 5 min. The colorless mixture suggested a negative reaction, that is, the reaction was complete.

(5) After the product obtained by the above reaction was sequentially washed with DMF (15 $mL \cdot g^{-1}$, twice), methanol (15 $mL \cdot g^{-1}$, twice) and DMF (15 $mL \cdot g^{-1}$, twice), the solvent was removed. 20% piperidine/DMF solution (10 $mL \cdot g^{-1}$) was added, and the solvent was removed after 5 min; 20% piperidine/DMF solution (10 $mL \cdot g^{-1}$) was added again, and the piperidine solution was removed after 15 min. A small amount of resin was taken and washed with ethanol, added with a ninhydrin reagent, and heated at 105–110 °C for 5 min. The color turned dark blue,

which suggested a positive reaction.

(6) After the product obtained by the above reaction was sequentially washed with DMF (15 $mL \cdot g^{-1}$, twice), methanol (15 $mL \cdot g^{-1}$, twice) and DCM (15 $mL \cdot g^{-1}$, twice), the resin was dried by suction.

(7) The product was cleavaged using a cleavaging liquid (15 $mL \cdot g^{-1}$, TFA:water:EDT:Tis = 95:1:2:2, v/v) for 90 min. The cleavaging fluid was blown to dryness with nitrogen, and then frozen to dryness to give a crude product of polypeptide.

(8) The polypeptide was purified and subjected to salt-conversion or desalting by HPLC. HPLC: tR = 6.1 mins (purification column model: Kromasil 100-5C18, 4.6 mm*250 mm; gradient eluent: acetonitrile with 0.1% TFA and aqueous solution with 0.1% TFA, 0 mins-1:99, 20 mins-1:9). The purified solution was frozen to dryness to give a purified product L-Thr-L-Pro (indicated as TP). The yield was about 80%. The mass spectrum presents $[M+H]^+$ at 217.3.

[Example 3] Synthesis of compound of formula (2)

(1) 2-chlorotrityl chloride resin was placed into a reaction tube, and added with DCM (20 $mL \cdot g^{-1}$). The resulting mixture was shaken for 30 min. With the use of a sand-core funnel by suction, the solvent was removed. The residue was added with a three-fold molar excess of Fmoc-L-Thr(tBu)-OH and an eight-fold molar excess of DIEA, and finally added with DMF to dissolve. The resulting mixture was shaken for 30 min. Methanol was used for end-capping for 30 min.

(2) The solvent DMF was removed. 20% piperidine/DMF solution (10 $mL \cdot g^{-1}$) was added, and the solvent was removed after 5 min; 20% piperidine/DMF solution (10 $mL \cdot g^{-1}$) was added again, and the piperidine solution was removed after 15 min. A small amount of resin was taken and washed with ethanol three times, added with a ninhydrin reagent, and heated at 105–110 °C for 5 min. The color turned dark blue, which suggested a positive reaction.

(3) After the product obtained by the above reaction was sequentially washed with DMF (15 $mL \cdot g^{-1}$, twice), methanol (15 $mL \cdot g^{-1}$, twice) and DMF (15 $mL \cdot g^{-1}$, twice), a two-fold excess of Fmoc-Arg(Pbf)-OH that was dissolved in as small an amount of DMF as possible was added to a reaction tube; a two-fold excess of HBTU was added. Immediately thereafter, an eight-fold excess of DIEA was added and reacted for 30 min.

(4) After the solution was removed by suction, a small amount of resin was taken and washed with ethanol three times, added with a ninhydrin reagent, and heated at 105–110 °C for 5 min. The colorless mixture suggested a negative reaction, that is, the reaction was complete.

(5) After the product obtained by the above reaction was sequentially washed with DMF (15 $mL \cdot g^{-1}$, twice), methanol (15 $mL \cdot g^{-1}$, twice) and DMF (15 $mL \cdot g^{-1}$, twice), the solvent was removed. 20% piperidine/DMF solution (10 $mL \cdot g^{-1}$) was added, and the solvent was removed after 5 min; 20% piperidine/DMF solution (10 $mL \cdot g^{-1}$) was added again, and the piperidine

solution was removed after 15 min. A small amount of resin was taken and washed with ethanol, added with a ninhydrin reagent, and heated at 105–110 °C for 5 min. The color turned dark blue, which suggested a positive reaction.

(6) After the product obtained by the above reaction was sequentially washed with DMF (15 mL·g^{-1}, twice), methanol (15 mL·g^{-1}, twice) and DCM (15 mL·g^{-1}, twice), the resin was dried by suction.

(7) The product was cleavaged using a cleavaging liquid (15 mL·g^{-1}, TFA:water:EDT:Tis = 95:1:2:2, v/v) for 90 min. The cleavaging fluid was blown to dryness with nitrogen, and then frozen to dryness to give a crude product of polypeptide.

(8) The polypeptide was purified and subjected to salt-conversion or desalting by HPLC. HPLC: tR = 4.8 mins (purification column model: Kromasil 100-5C18, 4.6 mm*250 mm; gradient eluent: acetonitrile with 0.1% TFA and aqueous solution with 0.1% TFA, 0 mins-1:99, 20 mins-1:4). The purified solution was frozen to dryness to give a purified product L-Thr-L-Arg (TR). The yield was about 80%. The mass spectrum presents $[M+H]^+$ at 276.2.

[Example 4] Synthesis of compound of formula (3)

(1) 2-chlorotrityl chloride resin was placed into a reaction tube, and added with DCM (20 mL·g^{-1}). The resulting mixture was shaken for 30 min. With the use of a sand-core funnel by suction, the solvent was removed. The residue was added with a three-fold molar excess of Fmoc-L-Thr(tBu)-OH and an eight-fold molar excess of DIEA, and finally added with DMF to dissolve. The resulting mixture was shaken for 30 min. Methanol was used for end-capping for 30 min.

(2) The solvent DMF was removed. 20% piperidine/DMF solution (10 mL·g^{-1}) was added, and the solvent was removed after 5 min; 20% piperidine/DMF solution (10 mL·g^{-1}) was added again, and the piperidine solution was removed after 15 min. A small amount of resin was taken and washed with ethanol three times, added with a ninhydrin reagent, and heated at 105–110 °C for 5 min. The color turned dark blue, which suggested a positive reaction.

(3) After the product obtained by the above reaction was sequentially washed with DMF (15 mL·g^{-1}, twice), methanol (15 mL·g^{-1}, twice) and DMF (15 mL·g^{-1}, twice), a two-fold excess of Fmoc-Arg(Pbf)-OH that was dissolved in as small an amount of DMF as possible was added to a reaction tube; a two-fold excess of HBTU was added. Immediately thereafter, an eight-fold excess of DIEA was added and reacted for 30 min.

(4) After the solution was removed by suction, a small amount of resin was taken and washed with ethanol three times, added with a ninhydrin reagent, and heated at 105–110 °C for 5 min. The colorless mixture suggested a negative reaction, that is, the reaction was complete.

(5) After the product obtained by the above reaction was sequentially washed with DMF (15 mL·g^{-1}, twice), methanol (15 mL·g^{-1}, twice) and DMF (15 mL·g^{-1}, twice), the solvent was

removed. 20% piperidine/DMF solution (10 mL·g^{-1}) was added, and the solvent was removed after 5 min; 20% piperidine/DMF solution (10 mL·g^{-1}) was added again, and the piperidine solution was removed after 15 min. A small amount of resin was taken and washed with ethanol, added with a ninhydrin reagent, and heated at 105–110 °C for 5 min. The color turned dark blue, which suggested a positive reaction.

(6) After the product obtained by the above reaction was sequentially washed with DMF (15 mL·g^{-1}, twice), methanol (15 mL·g^{-1}, twice) and DMF (15 mL·g^{-1}, twice), the resin was dried by suction.

(7) Steps (3) to (5) were repeated to link amino acid Fmoc-L-Thr(tBu)-OH. After the product obtained by the reaction was sequentially washed with DMF (15 mL·g^{-1}, twice), methanol (15 mL·g^{-1}, twice) and DCM (15 mL·g^{-1}, twice), the resin was dried by suction.

(8) The product was cleavaged using a cleavaging liquid (15 mL·g^{-1}, TFA:water:EDT:Tis = 95:1:2:2, v/v) for 90 min. The cleavaging fluid was blown to dryness with nitrogen, and then frozen to dryness to give a crude product of polypeptide.

(9) The polypeptide was purified and subjected to salt-conversion or desalting by HPLC. HPLC: tR = 3.9 mins (purification column model: Kromasil 100-5C18, 4.6 mm*250 mm; gradient eluent: acetonitrile with 0.1% TFA and aqueous solution with 0.1% TFA, 0 mins-1:99, 20 mins-1:4). The purified solution was frozen to dryness to give a purified product L-Thr-L-Arg-L-Thr (TRT). The yield was about 75%. The mass spectrum presents $[M+H]^+$ at 377.4.

[Example 5] Synthesis of compound of formula (4)

(1) 2-chlorotrityl chloride resin was placed into a reaction tube, and added with DCM (20 mL·g^{-1}). The resulting mixture was shaken for 30 min. With the use of a sand-core funnel by suction, the solvent was removed. The residue was added with a three-fold molar excess of Fmoc-L-Thr(tBu)-OH and an eight-fold molar excess of DIEA, and finally added with DMF to dissolve. The resulting mixture was shaken for 30 min. Methanol was used for end-capping for 30 min.

(2) The solvent DMF was removed. 20% piperidine/DMF solution (10 mL·g^{-1}) was added, and the solvent was removed after 5 min; 20% piperidine/DMF solution (10 mL·g^{-1}) was added again, and the piperidine solution was removed after 15 min. A small amount of resin was taken and washed with ethanol three times, added with a ninhydrin reagent, and heated at 105–110 °C for 5 min. The color turned dark blue, which suggested a positive reaction.

(3) After the product obtained by the above reaction was sequentially washed with DMF (15 mL·g^{-1}, twice), methanol (15 mL·g^{-1}, twice) and DMF (15 mL·g^{-1}, twice), a two-fold excess of Fmoc-L-Pro-OH that was dissolved in as small an amount of DMF as possible was added to a reaction tube; and a two-fold excess of HBTU was added. Immediately thereafter, an eight-fold excess of DIEA was added and reacted for 30 min.

(4) After the solution was removed by suction, a small amount of resin was taken and washed with ethanol three times, added with a ninhydrin reagent, and heated at 105–110 °C for 5 min. The colorless mixture suggested a negative reaction, that is, the reaction was complete.

(5) After the product obtained by the above reaction was sequentially washed with DMF (15 mL·g^{-1}, twice), methanol (15 mL·g^{-1}, twice) and DMF (15 mL·g^{-1}, twice), the solvent was removed. 20% piperidine/DMF solution (10 mL·g^{-1}) was added, and the solvent was removed after 5 min; 20% piperidine/DMF solution (10 mL·g^{-1}) was added again, and the piperidine solution was removed after 15 min. A small amount of resin was taken and washed with ethanol, added with a ninhydrin reagent, and heated at 105–110 °C for 5 min. The color turned dark blue, which suggested a positive reaction.

(6) After the product obtained by the above reaction was sequentially washed with DMF (15 mL·g^{-1}, twice), methanol (15 mL·g^{-1}, twice) and DMF (15 mL·g^{-1}, twice), the resin was dried by suction.

(7) Steps (3) to (5) were repeated to link amino acid Fmoc-L-Thr(tBu)-OH. After the product obtained by the reaction was sequentially washed with DMF (15 mL·g^{-1}, twice), methanol (15 mL·g^{-1}, twice) and DCM (15 mL·g^{-1}, twice), the resin was dried by suction.

(8) The product was cleavaged using a cleavaging liquid (15 mL·g^{-1}, TFA:water:EDT:Tis = 95:1:2:2, v/v) for 90 min. The cleavaging fluid was blown to dryness with nitrogen, and then frozen to dryness to give a crude product of polypeptide.

(9) The polypeptide was purified and subjected to salt-conversion or desalting by HPLC. HPLC: tR = 7.6 mins (purification column model: Kromasil 100-5C18, 4.6 mm*250 mm; gradient eluent: acetonitrile with 0.1% TFA and aqueous solution with 0.1% TFA, 0 mins-1:99, 20 mins-2:8). The purified solution was frozen to dryness to give a purified product L-Thr-L-Pro-L-Thr (TPT). The yield was about 70%. The mass spectrum presents $[M+H]^+$ at 318.3.

[Example 6] Synthesis of compound of formula (5)

(1) 2-chlorotrityl chloride resin was placed into a reaction tube, and added with DCM (20 mL·g^{-1}). The resulting mixture was shaken for 30 min. With the use of a sand-core funnel by suction, the solvent was removed. The residue was added with a three-fold molar excess of Fmoc-L-Thr(tBu)-OH and an eight-fold molar excess of DIEA, and finally added with DMF to dissolve. The resulting mixture was shaken for 30 min. Methanol was used for end-capping for 30 min.

(2) The solvent DMF was removed. 20% piperidine/DMF solution (10 mL·g^{-1}) was added, and the solvent was removed after 5 min; 20% piperidine/DMF solution (10 mL·g^{-1}) was added again, and the piperidine solution was removed after 15 min. A small amount of resin was taken and washed with ethanol three times, added with a ninhydrin reagent, and heated at 105–110 °C for 5 min. The color turned dark blue, which suggested a positive reaction.

(3) After the product obtained by the above reaction was sequentially washed with DMF (15 mL·g^{-1}, twice), methanol (15 mL·g^{-1}, twice) and DMF (15 mL·g^{-1}, twice), a two-fold excess of Fmoc-L-Ala-OH that was dissolved in as small an amount of DMF as possible was added to a reaction tube; a two-fold excess of HBTU was added. Immediately thereafter, an eight-fold excess of DIEA was added and reacted for 30 min.

(4) After the solution was removed by suction, a small amount of resin was taken and washed with ethanol three times, added with a ninhydrin reagent, and heated at 105–110 °C for 5 min. The colorless mixture suggested a negative reaction, that is, the reaction was complete.

(5) After the product obtained by the above reaction was sequentially washed with DMF (15 mL·g^{-1}, twice), methanol (15 mL·g^{-1}, twice) and DMF (15 mL·g^{-1}, twice), the solvent was removed. 20% piperidine/DMF solution (10 mL·g^{-1}) was added, and the solvent was removed after 5 min; 20% piperidine/DMF solution (10 mL·g^{-1}) was added again, and the piperidine solution was removed after 15 min. A small amount of resin was taken and washed with ethanol, added with a ninhydrin reagent, and heated at 105–110 °C for 5 min. The color turned dark blue, which suggested a positive reaction.

(6) After the product obtained by the above reaction was sequentially washed with DMF (15 mL·g^{-1}, twice), methanol (15 mL·g^{-1}, twice) and DMF (15 mL·g^{-1}, twice), the resin was dried by suction.

(7) Steps (3) to (5) were repeated to link amino acid Fmoc-L-Ala-OH. After the product obtained by the reaction was sequentially washed with DMF (15 mL·g^{-1}, twice), methanol (15 mL·g^{-1}, twice) and DCM (15 mL·g^{-1}, twice), the resin was dried by suction.

(8) The product was cleavaged using a cleavaging liquid (15 mL·g^{-1}, TFA:water:EDT:Tis = 95:1:2:2, v/v) for 90 min. The cleavaging fluid was blown to dryness with nitrogen, and then frozen to dryness to give a crude product of polypeptide.

(9) The polypeptide was purified and subjected to salt-conversion or desalting by HPLC. HPLC: tR = 7.9 mins (purification column model: Kromasil 100-5C18, 4.6 mm*250 mm; gradient eluent: acetonitrile with 0.1% TFA and aqueous solution with 0.1% TFA, 0 mins-1:99, 20 mins-1:9). The purified solution was frozen to dryness to give a purified product L-Ala-L-Ala-L-Thr (AAT). The yield was about 70%. The mass spectrum presents $[M-8H]^+$ at 260.1.

[Example 7] Synthesis of compounds of formula (6), formula (7) and formula (8)

Preparation of a compound of formula (6):

(1) GDL-L-Thr was prepared by using a solid-phase synthesis method.

(2) Purification by HPLC. HPLC: tR = 3.4 mins (purification column type: SHIMADZU Intertsil ODS-SP (4.6 mm*250 mm*5 μM), gradient eluent: acetonitrile with 0.1% TFA and aqueous solution with 0.1% TFA, 0.01–20 mins-1:99, 20–30 mins-21:79, 30–40 mins-100:0, 40–50 mins-

1:99); the yield was about 50%. The mass spectrum presents $[M-H]^+$ at 296.099.

The GDL-L-Thr prepared by using the solid-phase synthesis method has higher purity, and is more easily to separate. The experimental results show that the GDL-L-Thr prepared by using the solid-phase synthesis method has higher purity and good capability of inhibiting the growth of ice crystals (FIG. 13).

Compounds of both formulas (7) and (8) can be obtained by using a solid-phase synthesis method.

[Example 8] Synthesis of compound of formula (9)

(1) A DCM solution of dichlorodimethylisilane was poured into a synthesis tube for polypeptide, and after standing for 30 min the tube was air-dried for later use.

(2) 100 mg of resin was placed into the synthesis tube, 2 mL of DMF was added, and nitrogen was introduced. The resin was swollen for 10 min and filtered by suction under vacuum.

(3) 1mL of 4-methylpiperidine/DMF solution was added for deprotection, and removed after 5 min. 1 mL of 4-methylpiperidine/DMF solution was added again, and removed after 15 min. The mixture was bubbled and filtered by suction under vacuum.

(4) The mixture was washed with DMF 5 times, bubbled and filtered by suction under vacuum.

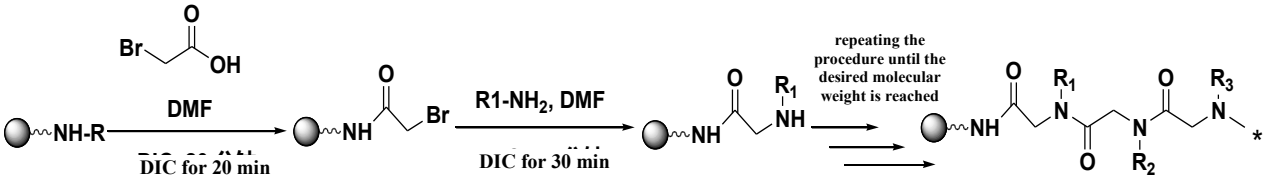
(5) The mixture was sequentially added with 0.5 mL of 2 M bromoacetic acid/DMF solution and *N*,*N*-diisopropylcarbodiimide/DMF solution, bubbled for 20 min, filtered by suction under vacuum, and washed with DMF 3 times.

(6) The mixture was added with 1 mL of 1 M primary amine/DMF solution, bubbled for 30 min, washed with DMF, and washed with dichloromethane (×3).

(7) Steps (5) and (6) were repeated until the desired molecular weight was reached.

(8) The mixture was added with 4 mL of a cracking liquid, homogeneously mixed, blown to dryness with nitrogen, finally frozen to dryness, and purified to give the purified product.

In a peptoid, R is $-CH_3$, $-CH_2CH_3$ and $-CH_2CH_2COOH$. The mass spectrum presents $[M+H]^+$ with R being $-CH_3$ at 444.6, $[M+H]^+$ with R being $-CH_2CH_3$ at 528.8, and $[M+H]^+$ with R being $-CH_2CH_2COOH$ at 792.1.



[Ice recrystallization inhibition experiment]

The IRI activity was assessed using “splat-freezing method”, wherein a sample was dissolved and dispersed into a DPBS solution, and 10–30 μL of the resultant solution was added dropwise onto the surface of a clean silicon disk precooled at -60 °C at a height of no less than 1.0 m; the solution was slowly heated to -6 °C at a speed of 10 °C/min by using a hot-cold stage, and was annealed for 30 min at this temperature; the sizes of ice crystals were observed and recorded by using a polarizing microscope and a high-speed camera. The hot-cold stage was sealed to ensure that the internal humidity was about 50%. The procedure was repeated at least three times for each sample, and the sizes of ice crystals were counted using a Nano Measurer 1.2, with the error of the result being the standard deviation.

DIS observation and TH measurement were performed by using a nanoliter osmometer. A capillary was first melt with the outer flame of a alcohol burner, and simultaneously stretched to produce a capillary with a very fine pore size, and the capillary was then linked to a microsyringe; an immersion oil with higher viscosity was injected into a disk with micron-sized holes, and an aqueous solution in which the material was dissolved was injected into the microholes by using a microsyringe; the droplet was quickly frozen by regulating the temperature, and then slowly heated to give a single crystal ice, the single crystal ice was slowly cooled at the precision of 0.01 °C, and the DIS observation and TH test were performed by using a microscope provided with a high-speed camera.

An IRI activity test was performed on 20 μL of a DPBS solution of the TR prepared in Example 3 by using "splat-freezing method". The determined MLGS (%) relative to that of DPBS is shown in FIG. 17. The MLGS of the TR bound by chemical bonds is obviously smaller than that of the DPBS solutions of arginine and threonine at the same concentration.

The deionized aqueous solution of the TR prepared in Example 3 was taken for DIS observation using a nanoliter osmometer. It was found that the TR had a weak modification effect on the topography of ice crystals (supercooling degree of -0.1 °C, -0.4 to 0.01 °C), as shown in FIG. 18. No TH was determined.

IRI activity tests were performed on 20 μL of DPBS solutions of the GDL-L-Thr, GDL-L-Ser and GDL-L-Val prepared in Example 7 by using "splat-freezing method". The determined MLGS (%) relative to that of DPBS is shown in FIG. 13 and FIGs. 15–16. The MLGSs of the GDL-L-Thr, GDL-L-Ser and GDL-L-Val bound by chemical bonds are obviously smaller than those of the DPBS solutions of GDL and amino acids at the same concentration, and small than those of the DPBS solutions of mixtures of GDL and amino acids at the same concentration.

The deionized aqueous solution of the GDL-L-Thr prepared in Example 7 was taken for DIS observation using a nanoliter osmometer. It was found that the GDL-L-Thr had a weak

modification effect on the topography of ice crystals (supercooling degree of -0.1 °C, -0.4 to 0.01 °C), as shown in FIG. 14. No TH was determined.

IRI activity tests were performed on 20 μL of DPBS solutions of the compounds prepared in Example 8 by using "splat-freezing method". The determined MLGS (%) relative to that of DPBS is shown in FIG. 19.

The deionized aqueous solutions of the three peptoids prepared in Example 8 were taken for DIS observation using a nanoliter osmometer. It was found that the peptoids where R was $-CH_3$ and $-CH_2CH_3$ had rather obvious modification effects on the topography of ice crystals, and the peptoids where R was $-CH_2CH_2COOH$ had no modification effect on the topography of ice crystals (supercooling degree of -0.1 °C, -0.4 to 0.01 °C). The topography obtained are shown in FIG. 20, and no TH was detected for the three peptoids.

The above results show that the prepared peptidic compounds have the activity to inhibit the growth of ice crystals and have modification effects on the topography of ice crystals, particularly the compound of formula (9) where R is $-CH_3$ or $-CH_2CH_3$ has an excellent modification effect on the topography of ice crystals with no TH, and can achieve the effect of controlling the growth of ice crystals and be used in the cryopreservation solution.

B. Ice growth inhibition performance evaluation and screening of ice growth inhibition material

The amount of the ice growth inhibition material adsorbed on an ice surface = (the mass of ice growth inhibition material adsorbed on ice surface m_1/total mass of ice growth inhibition material in stock solution comprising ice growth inhibition material m_2) × 100%.

In one embodiment, the ice adsorption experiment comprises the following steps:

S1, taking an ice growth inhibition material of the mass m_2 to prepare an aqueous solution of the ice control material, and cooling to a supercooling temperature;

S2, placing a pre-cooled temperature-regulating rod into the aqueous solution to induce the growth of an ice layer on the surface of the temperature-regulating rod, continuously stirring the aqueous solution to enable the ice growth inhibition material to be gradually adsorbed onto the surface of the ice layer, and keeping the temperature of the aqueous solution and the temperature-regulating rod at a supercooling temperature; and

S3, determining the amount of the ice growth inhibition material absorbed on the ice surface.

The device shown in FIG. 21 was used to perform the ice adsorption experiment. The device comprises a multilayer liquid storage cavity, a temperature-regulating rod and a temperature regulator, wherein the multilayer liquid storage cavity sequentially comprises an ice adsorption cavity, a bath cavity and a cooling liquid storage cavity from the inside to the outside, the temperature-regulating rod being arranged in the ice adsorption cavity, and the temperatures of the temperature-regulating rod and the liquid storage cavity being regulated by the temperature regulator. The temperature-

regulating rod is of a hollow structure made of a thermally conductive material, and the hollow structure of the temperature-regulating rod is provided with a liquid inlet and a liquid outlet; the temperature regulator is a fluid temperature regulator and is provided with a cooling liquid outflow end and a reflux end; two ends of the cooling liquid storage cavity is provided with a liquid inlet and a liquid outlet; the cooling liquid outflow end of the temperature regulator, the liquid inlet of the temperature-regulating rod, the liquid outlet of the temperature-regulating rod, the liquid inlet of a cooling liquid storage tank, the liquid outlet of the cooling liquid storage tank and the reflux end of the temperature regulator are sequentially linked via pipelines through which the cooling liquid flows. The multilayer liquid storage cavity is provided with a cover. When in use, the ice adsorption cavity is arranged to contain an aqueous solution of the ice growth inhibition material, and the bath cavity in the middle layer is arranged to contain a bath medium that is at a preset temperature, for example, a water bath, an ice bath or an oil bath; after the preset temperature of the cooling liquid is reached, the cooling liquid flows out through the temperature regulator and flows into the hollow structure of the temperature-regulating rod to regulate the temperature of the temperature-regulating rod, then flows out from the liquid outlet of the temperature-regulating rod and flows into the cooling liquid storage cavity in the outer layer to maintain the temperature of the bath medium at the preset level, and then flows through the liquid outlet of the cooling liquid storage tank and the reflux end of the temperature regulator and enters the temperature regulator to circulate.

[Example 9]

a-PVA: molecular weight of about 13–23 kDa, diad syndiotacticity r of about 55% (Sigma-Aldrich);

i-PVA: molecular weight of about 14–26 kDa, isotacticity m of about 84%.

(1) Measurement of spreading performance of two PVAs at ice-water interface

The amount of the PVAs adsorbed on an ice surface was determined by performing an ice adsorption experiment, and an experimental device is shown in FIG 21.

a. The a-PVA and the i-PVA were fluorescently labeled with FITC Isomer I.

b. The FITC-labeled aqueous solutions (40 mL) of the PVAs with different concentrations were placed in beakers, which were then placed in a recirculating cooling bath, and the temperatures of the solutions and the temperature-regulating rod were cooled to -0.1 °C.

c. The temperature-regulating rod was inserted into liquid nitrogen for pre-cooling for 1.0 min before being inserted into the pre-cooled FITC-labeled aqueous solutions of the PVAs. Then, the temperature-regulating rod was rapidly inserted into the pre-cooled FITC-labeled aqueous solutions of the PVAs to induce an extremely thin ice layer on the surface of the temperature-regulating rod to further induce ice growth.

d. The aqueous FITC-labeled PVA solution was magnetically stirred continuously at a

supercooling temperature of -0.1 °C for 1.0 h to allow the PVA to be gradually adsorbed onto the surface of the ice. The supercooling degree and the adsorption time were maintained unchanged during all adsorption experiments to ensure that the surface area of the resulting ice was almost maintained unchanged within an allowable error range.

e. The formed ice block was taken out from the solution, and the ice surface was rinsed with purified water to remove the solution attached to the surface. The ice block was melted.

f. The amount of the PVA absorbed on the ice surface was obtained by the mass ratio of the solute PVA in the ice block to the solute PVA in the original solution, the concentration of the PVA solution was determined by ultraviolet-visible spectrophotometry, and the volume was determined by a pipette and a measuring cylinder.

In ice adsorption experiments, the amounts of the a-PVA and the i-PVA absorbed with each concentration are shown in FIG. 22, wherein the amount of the a-PVA absorbed on the ice surface increases from 16.3% when the concentration is 0.2 $mg \cdot mL^{-1}$ to 28.7% when the concentration is 1.0 $mg \cdot mL^{-1}$, and the amount of the a-PVA absorbed on the ice surface is saturated after the concentration is more than 1.0 mg mL^{-1} with the adsorption amount of about 36.5%. The amount of the i-PVA absorbed on the ice surface is 0%–19.3% when the concentration is less than 1.0 $mg \cdot mL^{-1}$, and is lower than that of the a-PVA absorbed on the ice surface at the same concentration. At low concentrations, the amounts of the two PVAs adsorbed on the ice surface are not saturated, and the ice surface area covered by the i-PVA is lower than that of the a-PVA. The amount of the i-PVA adsorbed on the ice surface is higher than that of the a-PVA when the concentration of the i-PVA is more than or equal to 1.2 $mg \cdot mL^{-1}$, and the amount of the i-PVA absorbed on the ice surface is saturated when the concentration is 2.0 $mg \cdot mL^{-1}$ with the adsorption amount of 56.5%. Further, it is stated that the amount of the i-PVA required is much greater than that of the a-PVA when the amounts of the two PVAs absorbed on ice surfaces with the same size are saturated. That is, the a-PVA could more effectively cover the surface of ice.

(2) Assay for ice recrystallization inhibition (IRI) activity

The ice recrystallization inhibition (IRI) activity was assessed using “splat-freezing method”, wherein the two PVAs were separately dissolved and dispersed into DPBS solutions, and 10–30 μL of the resulting solution was added dropwise onto the surface of a clean silicon disk pre-cooled at -60 °C at a height of no less than 1.0 m; the solution was slowly heated to -6 °C at a speed of 10 $°C \cdot min^{-1}$ by using a hot-cold stage, and was annealed for 30 min at this temperature; the sizes of ice crystals were observed and recorded by using a polarizing microscope and a high-speed camera. The hot-cold stage was sealed to ensure that the internal humidity was about 50%. The procedure was repeated at least three times for each sample, and the size of the ice crystal was counted using a Nano Measurer 1.2, with the error of the result being the standard deviation.

The result is shown in FIG. 23 where the grain size of the a-PVA was significantly smaller than that of the i-PVA at the same concentration, which indicates that the ability of the a-PVA to inhibit the growth of ice crystals is far superior to that of the i-PVA.

According to the results of Example 9, the i-PVA has a weaker affinity for water than the a-PVA. Therefore, the i-PVA tends to exist in an aggregated state in an aqueous solution and on an ice-water interface, while the a-PVA can be well spread in an aqueous solution and on an ice-water interface. The amount of the i-PVA required is much higher than that of the a-PVA when the amounts of the two PVAs absorbed on ice surfaces with the same size are saturated. Therefore, compared with the i-PVA, the a-PVA is a better ice growth inhibition material, playing a better role in inhibiting the growth of ice crystals at lower concentrations.

C. Formulation of cryopreservation solution and preparation and application embodiments

[Example 10] Preparation of cryopreservation solution comprising PVA as ice growth inhibition material

1. Preparation of cryopreservation solutions: cryopreservation solutions were prepared according to the following formulations.

A cryopreservation solution A comprises the following components per 100 mL:

Substances	Content
PVA (g)	2.0
Ethylene glycol (mL)	10
DMSO (mL)	10
Sucrose ($mol \cdot L^{-1}$)	0.5
Fetal bovine serum (mL)	20
DPBS (mL)	Balance

Solution preparation steps: 2.0 g of a PVA was dissolved in 25 mL of DPBS in a water bath at 80 °C by heating magnetic stirring, and pH was adjusted to 7.0 to give a solution 1 after the PVA was completely dissolved and cooled to the room temperature; 17 g (0.05 mol) of sucrose (the final concentration of the sucrose in the cryopreservation solution was 0.5 $mol \cdot L^{-1}$) was ultrasonically dissolved in 25 mL of DPBS, and after the sucrose was completely dissolved, 10 mL of ethylene glycol and 10 mL of DMSO were added to give a solution 2; after returning to room temperature, the solution 1 and the solution 2 were homogeneously mixed, the pH was adjusted, the volume was made up to 80%, and 20 mL of serum was stored separately to be added

when the cryopreservation solution was used.

A cryopreservation solution B comprises the following components per 100 mL:

Substances	Content
L-Arg (g)	8.0
L-Thr (g)	4.0
PVA (g)	2.0
Ethylene glycol (mL)	10
Sucrose ($mol \cdot L^{-1}$)	0.5
Fetal bovine serum (mL)	20
DPBS (mL)	Balance

Solution preparation steps: 2.0 g of a PVA was dissolved in 20 mL of DPBS in a water bath at 80 °C by heating magnetic stirring, and pH was adjusted to 7.1 to give a solution 1; 8.0 g of L-Arg and 4.0 g of L-Thr were dissolved in 20 mL of DPBS, and the pH was adjusted to 7.1 to give a solution 2; 17 g (0.05 mol) of sucrose (the final concentration of the sucrose in the cryopreservation solution was 0.5 $mol \cdot L^{-1}$) was ultrasonically dissolved in 20 mL of DPBS, and after the sucrose was completely dissolved, 10 mL of ethylene glycol was added to give a solution 3; after returning to room temperature, the solution 1, the solution 2 and the solution 3 were homogeneously mixed, the pH was adjusted, the volume was made up to 80%, and 20 mL of serum was added when the cryopreservation was used.

A cryopreservation solution C comprises the following components per 100 mL:

Substances	Content
PVA (g)	2.0
Ethylene glycol (mL)	10
Sucrose ($mol \cdot L^{-1}$)	0.5
Fetal bovine serum (mL)	20
DPBS (mL)	Balance

Solution preparation steps: 2.0 g of a PVA was dissolved in 25 mL of DPBS in a water bath at 80 °C by heating magnetic stirring, and pH was adjusted to 6.9 to give a solution 1; 17 g (0.05 mol) of sucrose (the final concentration of the sucrose in the cryopreservation solution was 0.5 $mol \cdot L^{-1}$) was ultrasonically dissolved in 25 mL of DPBS, and after the sucrose was completely dissolved, 10 mL of ethylene glycol was added to give a solution 2; after returning to room temperature, the solution 1 and the solution 2 were homogeneously mixed, the pH was adjusted, the volume was made up to 80%, and 20 mL of serum was stored separately to be added when the cryopreservation solution was used.

A cryopreservation solution C1 comprises the following components per 100 mL:

Substances	Content
PVA (g)	1.0
Ethylene glycol (mL)	10
Sucrose ($mol \cdot L^{-1}$)	0.5
Serum (mL)	20
DPBS (ml)	Balance

The solution preparation steps were the same as those of the cryopreservation solution C.

A cryopreservation solution D comprises the following components per 100 mL:

Substances	Content
PVA (g)	2.0
Ethylene glycol (mL)	10
Sucrose ($mol \cdot L^{-1}$)	0.5
DPBS (mL)	Balance

Solution preparation steps: 2.0 g of a PVA was dissolved in 30 mL of DPBS in a water bath at 80 °C by heating magnetic stirring, and pH was adjusted to 7.0 to give a solution 1; 17 g (0.05 mol) of sucrose (the final concentration of the sucrose in the cryopreservation solution was 0.5 $mol \cdot L^{-1}$) was ultrasonically dissolved in 25 mL of DPBS, and after the sucrose was completely dissolved, 10 mL of ethylene glycol was added to give a solution 2; after returning to room temperature, the solution 1 and the solution 2 were homogeneously mixed, the pH was adjusted, and the volume

was made up to 100 mL for later use.

A cryopreservation solution E comprises the following components per 100 mL:

Substances	Content
Poly-L-proline (g)	1.5
PVA (g)	2.0
Ethylene glycol (mL)	10
Sucrose ($mol \cdot L^{-1}$)	0.5
DPBS (ml)	Balance

Solution preparation steps: 2.0 g of a PVA was dissolved in 25 mL of DPBS in a water bath at 80 °C by heating magnetic stirring, and pH was adjusted to 7.0 to give a solution 1; 1.5 g of poly-L-proline (with the degree of polymerization of 15) was ultrasonically dissolved in another 20 mL of DPBS, and the pH was adjusted to 7.0 to give a solution 2; 17 g (0.05 mol) of sucrose (the final concentration of the sucrose in the cryopreservation solution was 0.5 $mol \cdot L^{-1}$) was ultrasonically dissolved in 25 mL of DPBS, and after the sucrose was completely dissolved, 10 mL of ethylene glycol was added to give a solution 3; after returning to room temperature, the solution 1, the solution 2 and the solution 3 were homogeneously mixed, the pH was adjusted, and the volume was made up to 100 mL for later use.

The cryopreservation solution F comprises the following components per 100 mL:

Substances	Content
Poly-L-arginine (g, degree of polymerization being 8)	4.0
PVA (g)	1.0
Ethylene glycol (mL)	10
Sucrose ($mol \cdot L^{-1}$)	0.5
Serum (mL)	20
DPBS (ml)	Balance

The solution preparation steps were the same as those of the cryopreservation solution E, and the serum was added when the cryopreservation solution was used.

2. Preparation of freezing equilibration solutions: the freezing equilibration solutions were prepared according to the following formulations.

A freezing equilibration solution a: 7.5 mL of ethylene glycol and 7.5 mL of DMSO were added to 65 mL of DPBS, and mixed homogeneously, and 20 mL of serum was added when the freezing equilibration solution was used.

A freezing equilibration solution b: 7.5 mL of ethylene glycol was dissolved in 72.5 mL of DPBS, and mixed homogeneously, and 20 mL of serum was added when the freezing equilibration solution was used.

A freezing equilibration solution c: 2.0 g of a PVA was dissolved in 50 mL of DPBS in a water bath at 80 °C by heating magnetic stirring, pH was adjusted to 7.0 after the PVA was completely dissolved, 7.5 mL of ethylene glycol was added, and mixed homogeneously, the pH was adjusted, and the volume was made up to 100 mL for later use.

Comparative Example:

A freezing equilibration solution a comprises, per 1 mL, 7.5% (v/v) of DMSO, 7.5% (v/v) of ethylene glycol, 20% (v/v) of fetal bovine serum and the balance of DPBS;

A cryopreservation solution 1# comprises, per 1 mL, 15% (v/v) of DMSO, 15% (v/v) of ethylene glycol, 20% (v/v) of fetal bovine serum, 0.5 M sucrose and the balance of DPBS.

A freezing equilibration solution 2# comprises, per 1 mL, 7.5% (v/v) of ethylene glycol, 20% (v/v) of fetal bovine serum and the balance of DPBS;

A cryopreservation solution 2# comprises, per 1 mL, 10% (v/v) of ethylene glycol, 20% (v/v) of fetal bovine serum, 0.5 M sucrose and the balance of DPBS.

A cryopreservation solution 3# comprises, per 1 mL, 10% (v/v) of DMSO, 15% (v/v) of fetal bovine serum and the balance of a-MEM (USA, Invitrogen, C12571500BT).

The three formulations of the thawing solutions used in Example 10 and the comparative examples were as follows:

A thawing solution 1# comprises a thawing solution I (comprising sucrose at 1.0 $mol \cdot L^{-1}$, 20% of serum and the balance of DPBS), a thawing solution II (comprising sucrose at 0.5 $mol \cdot L^{-1}$, 20% of serum and the balance of DPBS), a thawing solution III (comprising sucrose at 0.25 $mol \cdot L^{-1}$, 20% of serum and the balance of DPBS), and a thawing solution IV (comprising 20% of serum and the balance of DPBS).

A thawing solution 2# comprises a thawing solution I (comprising sucrose at 1.0 $mol \cdot L^{-1}$, a PVA at 20 $mg \cdot mL^{-1}$ and the balance of DPBS), a thawing solution II (comprising sucrose at 0.5 $mol \cdot L^{-1}$, a PVA at 20 $mg \cdot mL^{-1}$ and the balance of DPBS), a thawing solution III (comprising sucrose at 0.25 $mol \cdot L^{-1}$, a PVA at 20 mg mL^{-1} and the balance of DPBS), and a thawing solution IV (comprising a PVA at 20 $mg \cdot mL^{-1}$ and the balance of DPBS).

A thawing solution 3# comprises a thawing solution I (comprising sucrose at 1.0 mol·L^{-1}, a PVA at 20 mg·mL^{-1}, 10 mg·mL^{-1} polyproline and the balance of DPBS), a thawing solution II (comprising sucrose at 0.5 mol·L^{-1}, a PVA at 20 mg·mL^{-1}, 5.0 mg·mL^{-1} polyproline and the balance of DPBS), a thawing solution III (comprising sucrose at 0.25 mol·L^{-1}, a PVA at 20 mg·mL^{-1}, 2.5 mg·mL^{-1} polyproline and the balance of DPBS), and a thawing solution IV (comprising a PVA at 20 mg·mL^{-1} and the balance of DPBS).

[Application Example 1] Oocytes and embryos were cryopreserved using the freezing equilibration solutions and the cryopreservation solutions of the above examples and comparative examples according to the schemes in Table 1 and Table 2, respectively. The survival rates in the embodiments of the present invention were the average survival rate of 3–12 repeated experiments.

1. Cryopreservation of oocytes

Mouse oocytes were firstly equilibrated in a freezing equilibration solution for 5 min, and then put in the prepared cryopreservation solution for 1 min; the equilibrated oocytes in the cryopreservation solution were placed on a straw, then quickly put into liquid nitrogen (-196 °C), and continuously preserved after the carrying rod was sealed; at the time of thawing, the frozen oocytes were equilibrated in the thawing solution I at 37 °C for 5 min, and equilibrated in the thawing solutions II–IV in sequence for 3 min each; and after the thawed oocytes were cultured for 2 h, the number of the survived cells was observed, and the survival rates were calculated (see Table 1).

2. Cryopreservation of embryos

Mouse embryos were firstly equilibrated in a freezing equilibration solution for 5 min, and then put into the cryopreservation solution prepared in accordance to the formulations of the above examples and comparative examples for 50 s; the equilibrated embryos in the cryopreservation solution were placed on a straw, then quickly put into liquid nitrogen (-196 °C) and continuously preserved after the carrying rod was sealed; at the time of thawing, the frozen embryos were equilibrated in the thawing solution I at 37 °C for 3 min, and then equilibrated in the thawing solutions II–IV in sequence for 3 min each; and after the thawed embryos were cultured for 2 h, the number of the survived embryos was observed, and the survival rates were calculated (see Table 2).

Table 1. Survival rates of cryopreserved mouse oocytes

No.	Equilibration solution	Cryopreservation solution	Thawing solution	Total number of frozen oocytes	Survival rates after 2 h
Application Embodiment 1	a	A	Thawing solution 1#	67	100.0%
Application Embodiment 2	b	B	Thawing solution 1#	109	94.8%
Application Embodiment 3	b	C	Thawing solution 1#	90	97.7%
Application Embodiment 4	c	D	Thawing solution 1#	50	93.4%
Application Embodiment 5	c	D	Thawing solution 2#	53	96.5%
Application Embodiment 6	c	E	Thawing solution 1#	39	89.7%
Application Embodiment 7	c	E	Thawing solution 3#	60	98.6%
Comparative Embodiment 1	a	Freezing solution 1#	Thawing solution 1#	146	95.0%
Comparative Embodiment 2	Equilibration solution 2#	Freezing solution 2#	Thawing solution 1#	96	81.9%
Comparative Embodiment 3	Equilibration solution 2#	Freezing solution 2#	Thawing solution 2#	44	94.7%

Table 2. Survival rates of cryopreserved mouse embryos

No.	Equilibration solution	Cryopreservation solution	Thawing solution	Total number of frozen embryos	Survival rates after 2 h
Application Embodiment 8	c	D	Thawing solution 1#	41	95.8%
Application Embodiment 9	c	E	Thawing solution 1#	42	95.2%
Comparative Embodiment 4	a	Freezing solution 1#	Thawing solution 1#	38	94.3%
Comparative Embodiment 5	Equilibration solution 2#	Freezing solution 2#	Thawing solution 1#	39	82.4%

The above data indicate that the survival rate of the cryopreservation solution can be no less than 90% and even 100%, and can reach or far exceed a cryopreservation thawing rate of a commercial cryopreservation solution comprising 15% DMSO and commonly available in clinical practice at present, and as can be seen from the comparison of Application Embodiment 1 (comprising 10% DMSO), Comparative Embodiment 2 (comprising 7.5% DMSO) and Comparative Embodiment 1 (namely commercial oocyte cryopreservation solution (comprising 15% DMSO)), the survival rate of oocytes is significantly improved by adding PVA; Application Embodiments 2–3 also show that the cryopreservation solution can have higher survival rates of oocytes or embryos by adding a small amount of DMSO or not adding DMSO, solving the problem that the DMSO concentration of commercial cryopreservation solutions commonly available in clinical practice is high and the damage to cells is large; moreover, Application Embodiments 5 and 7-9 show that higher survival rates of oocytes or embryos can be realized under the condition that DMSO and serum are not added in the freezing solutions, equilibration solutions and thawing solutions. The DMSO-free or serum-free cryopreservation solution solves the problems of short shelf life, introduction of parasitic biological pollutants and the like caused by serum comprised in the commercial cryopreservation solutions commonly available in clinical practice at present.

[Application Example 2] Cryopreservation of human umbilical cord mesenchymal stem cells

Human umbilical cord mesenchymal stem cells were cryopreserved using the cryopreservation solutions of the above examples and comparative examples according to the scheme in Table 3. Cryopreservation of human umbilical cord mesenchymal stem cells by microdroplet method: digesting human umbilical cord mesenchymal stem cells on a culture dish using 25% pancreatin for 2 min, putting the digested human umbilical cord mesenchymal stem cells into a culture solution (10% FBS + a-MEM culture medium) of the same volume, gently pipetting until the stem cells completely fall off, adding the cells into a 1.5 mL centrifuge tube for centrifuging for 5 min at 1000 rmp, discarding the supernatant (separating the cells from the culture medium), adding 10 μL of a freezing solution to the bottom of the centrifuge tube, gently pipetting to disperse stem cell clusters, placing the 10 μL of the freezing solution with the stem cells on a freezing slide, and cryopreserving the solution into liquid nitrogen (-196 °C). At the time of thawing, the straw with the cells and the freezing solution was placed directly in a culture medium at 37 °C for thawing. After thawing, cells were stained with trypan blue to observe the survival rates, and the number of cells was counted using an instrument JIMBIO-FIL, survival rate = number of live cells/total number of cells (see Table 3).

Table 3. Survival rates of cryopreserved human umbilical cord mesenchymal stem cells

No.	Cryopreservation solution	Cryopreservation method	Survival rates
Application Embodiment 10	C1	Microdroplet method	72.2%
Application Embodiment 11	D	Microdroplet method	77.1%
Application Embodiment 12	F	Microdroplet method	92.4%
Comparative Embodiment 6	Freezing solution 1#	Microdroplet method	63.9%
Comparative Embodiment 7	Freezing solution 3#	Microdroplet method	76.6%

When the cryopreservation solution disclosed herein is used for cryopreservation of the human umbilical cord mesenchymal stem cells, the survival rates of the stem cells can reach 92.4% and 72.2%, respectively (in Application Embodiments 12 and 10) although no DMSO is added, and the survival rate can even reach 77.1% when no DMSO and serum is added, reaching the survival level of the existing freezing regent. This means that the freezing reagent can have the same effectiveness in freezing stem cells as a conventional freezing solution, a cryopreservation thawing rate of the reagent can reach or even be far higher than that of a commonly available cryopreservation solution comprising 10% DMSO (in Comparative Embodiment 7), and the PVA-based cryopreservation effect is remarkably superior to the PVA-free cryopreservation effect in Comparative Embodiment 6.

[Application Example 3] Cryopreservation of ovarian organs and ovarian tissues

The ovarian organs of mice newly born within 3 days and the ovarian tissue slices of sexually mature mice were cryopreserved using the freezing equilibration solutions and cryopreservation solutions of the above examples and comparative examples according to the schemes in Table 4 and Table 5.

The intact ovarian organs or ovarian tissue slices were firstly equilibrated in an equilibration solution at room temperature for 25 min, and then put into the prepared cryopreservation solution for 15 min. Next, the intact ovarian organs or ovarian tissue slices were placed on a straw and put into liquid nitrogen for preservation. After being thawed, the intact ovarian organs or ovarian tissue slices were put into a culture solution (10% FBS + a-MEM) in an incubator at 37 °C in the presence of 5% CO_2 for 2 h for further thawing. Next, the intact ovarian organs or ovarian tissue slices were fixed with 4% paraformaldehyde, embedded in paraffin, and subjected to HE staining, and then the morphology was observed. The results are shown in FIGs. 24–33, wherein FIG. 24

is a picture of a slice of a fresh unfrozen ovarian organ, and FIG. 29 is a picture of a slice of fresh unfrozen ovarian tissue.

Table 4. Ovarian organ cryopreservation scheme

No.	Equilibration solution	Cryopreservation solution	Thawing solution	Morphology
Application Embodiment 13	c	D	Thawing solution 2#	FIG. 26
Application Embodiment 14	b	C1	Thawing solution 1#	FIG. 27
Application Embodiment 15	b	F	Thawing solution 1#	FIG. 28
Comparative Embodiment 8	a	Freezing solution 1#	Thawing solution 1#	FIG. 25

Table 5. Ovarian tissue cryopreservation scheme

No.	Equilibration solution	Cryopreservation solution	Thawing solution	Morphology
Application Embodiment 16	c	D	Thawing solution 2#	FIG. 31
Application Embodiment 17	b	C1	Thawing solution 1#	FIG. 32
Application Embodiment 18	b	F	Thawing solution 1#	FIG. 33
Comparative Embodiment 9	a	Freezing solution 1#	Thawing solution 1#	FIG. 30

As can be seen from FIGs. 24–28, compared with Comparative Embodiment 8 free of polyvinyl alcohol and fresh unfrozen ovarian organs, the schemes of Application Embodiments 13–15 are characterized in that: the original follicle structure is relatively intact, the interstitial structure is relatively intact, the cytoplasm of cells is relatively homogeneous and lightly stained in a relatively large amount, and nucleus shrinkage and deep staining were relatively mild; blood vessels have intact vessel wall structures and less luminal collapse, the cytoplasm of endothelial cells is relatively homogeneous and lightly stained in a relatively large amount, and nucleus shrinkage and deep staining were relatively mild. As can be seen, Application Embodiments 13–15 have better cryopreservation effects on ovarian organs.

As can be seen from FIGs. 29–33, compared with Comparative Embodiment 9 and fresh unfrozen ovarian tissues, the schemes of Application Embodiments 16–18 are characterized in that: the antral follicle structure is relatively intact, the interstitial structure is relatively intact, the cytoplasm is relatively homogeneous and lightly stained in a relatively large amount, and nucleus shrinkage and deep staining were relatively mild. It can be seen that the cryopreservation solution

disclosed herein has a better cryopreservation effect on ovarian tissues than the prior art.

It can be seen that the cryopreservation solution prepared with the biomimetic PVA-based ice growth inhibition material as a main component disclosed herein has a good inhibition effect on the growth of ice crystals, can be used with DMSO being reduced in the preservation system or even without DMSO, maintain good biocompatibility, and can be simultaneously applied to cryopreservation of oocytes, embryos, stem cells, reproductive organs and tissues where high cell survival rates and good biological activity can be achieved.

[Example 11] Preparation of cryopreservation solution comprising amino acid as ice growth inhibition material

A cryopreservation solution G comprises the following components per 100 mL:

Substances	Content
L-Arg (g)	16.0
L-Thr (g)	8.0
DMSO (mL)	10
Ethylene glycol (mL)	10
Sucrose ($mol \cdot L^{-1}$)	0.5
Fetal bovine serum (mL)	20
DPBS (mL)	Balance

Solution preparation steps (total volume: 100 mL): 16 g of L-Arg and 8 g of L-Thr were dissolved in 25 mL of DPBS, and pH was adjusted to 6.9 to give a solution 1; 17 g (0.05 mol) of sucrose (the final concentration of the sucrose in the cryopreservation solution was 0.5 $mol \cdot L^{-1}$) was ultrasonically dissolved in 25 mL of DPBS, and after the sucrose was completely dissolved, 10 mL of ethylene glycol and 10 mL of DMSO were sequentially added to give a solution 2; after returning to room temperature, the solution 1 and the solution 2 were homogeneously mixed, the pH was adjusted to 6.9, the volume was made up to 80% with DPBS, and 20 mL of fetal bovine serum was stored separately to be added before the cryopreservation solution was used.

A cryopreservation solution H comprises the following components per 100 mL:

Substances	Content
Poly-L-proline (g, degree of polymerization being 15)	1.5
DMSO (mL)	10
Ethylene glycol (mL)	10
Sucrose ($mol \cdot L^{-1}$)	0.5
Fetal bovine serum (mL)	20
DPBS (mL)	Balance

Solution preparation steps: 1.5 g of poly-L-proline (with the degree of polymerization of 15) was ultrasonically dissolved in 25 mL of DPBS, and pH was adjusted to 6.8 to give a solution 1; 17 g (0.05 mol) of sucrose was ultrasonically dissolved in 25 mL of DPBS, and after the sucrose was completely dissolved, 10 mL of ethylene glycol and 10 mL of DMSO were sequentially added to give a solution 2; after returning to room temperature, the solution 1 and the solution 2 were homogeneously mixed, the pH was adjusted to 7.0, the volume was made up to 80% with DPBS, and 20 mL of serum was stored separately to be added before the cryopreservation solution was used.

A cryopreservation solution I comprises the following components per 100 mL:

Substances	Content
Poly-L-arginine (g, degree of polymerization being 8)	1.5
DMSO (mL)	10
Ethylene glycol (mL)	10
Sucrose ($mol \cdot L^{-1}$)	0.5
Fetal bovine serum (mL)	20
DPBS (mL)	Balance

Solution preparation steps (total volume: 100 mL): 1.5 g of poly-L-arginine (with the degree of polymerization of 8) was ultrasonically dissolved in 25 mL of DPBS, and pH was adjusted to 7.0 to give a solution 1; 17 g (0.05 mol) of sucrose was ultrasonically dissolved in 20 mL of DPBS, and after the sucrose was completely dissolved, 10 mL of ethylene glycol and 10 mL of DMSO were sequentially added to give a solution 2; after returning to room temperature, the solution 1

and the solution 2 were homogeneously mixed, the pH was adjusted to 7.0, the volume was made up to 80% with DPBS, and 20 mL of serum was stored separately to be added before the cryopreservation solution was used.

A cryopreservation solution J comprises the following components per 100 mL:

Substances	Content
Poly-L-arginine (g, degree of polymerization being 8)	4.0
DMSO (mL)	7.5
Ethylene glycol (mL)	10
Sucrose ($mol \cdot L^{-1}$)	0.5
Fetal bovine serum (mL)	20
DPBS (mL)	Balance

The solution preparation steps were the same as those of the cryopreservation solution I.

A cryopreservation solution K comprises the following components per 100 mL:

Substances	Content
Poly-L-proline (g, degree of polymerization being 8)	4.0
DMSO (mL)	7.5
Ethylene glycol (mL)	10
Sucrose ($mol \cdot L^{-1}$)	0.5
Fetal bovine serum (mL)	20
DPBS (mL)	Balance

The solution preparation steps were the same as those of the cryopreservation solution I.

A cryopreservation solution L comprises the following components per 100 mL:

Substances	Content
L-Arg (g)	16.0
L-Thr (g)	8.0
DMSO (mL)	7.5
Ethylene glycol (mL)	10
Sucrose ($mol \cdot L^{-1}$)	0.5
Fetal bovine serum (mL)	20
DPBS (mL)	Balance

The solution preparation steps were the same as those of the cryopreservation solution G.

Preparation of freezing equilibration solutions: the freezing equilibration solutions were prepared according to the following formulations.

A freezing equilibration solution a: 7.5 mL of ethylene glycol and 7.5 mL of DMSO were added to 65 mL of DPBS, and mixed homogeneously, and 20 mL of serum was added when the freezing equilibration solution was used.

A freezing equilibration solution b: 7.5 mL of ethylene glycol was added to 72.5 mL of DPBS, and mixed homogeneously, and 20 mL of serum was added when the freezing equilibration solution was used.

Comparative Example 2:

A freezing equilibration solution a comprises, per 1 mL, 7.5% (v/v) of DMSO, 7.5% (v/v) of ethylene glycol, 20% (v/v) of fetal bovine serum and the balance of DPBS;

A cryopreservation solution 1# comprises, per 1 mL, 15% (v/v) of DMSO, 15% (v/v) of ethylene glycol, 20% (v/v) of fetal bovine serum, 0.5 M sucrose and the balance of DPBS.

A cryopreservation solution 3# comprises, per 1 mL, 10% (v/v) of DMSO, 15% (v/v) of fetal bovine serum and the balance of a-MEM (USA, Invitrogen, C12571500BT).

The formulation of the thawing solutions used in Example 11 and Comparative Example 2 was as follows:

A thawing solution 1# comprises a thawing solution I (comprising sucrose at $1.0 mol \cdot L^{-1}$, 20% of serum and the balance of DPBS), a thawing solution II (comprising sucrose at $0.5 mol \cdot L^{-1}$, 20% of serum and the balance of DPBS), a thawing solution III (comprising sucrose at $0.25 mol \cdot L^{-1}$, 20% of serum and the balance of DPBS), and a thawing solution IV (comprising 20% of serum and the balance of DPBS).

[Application Example 4] Cryopreservation of oocytes and embryos

Oocytes and embryos were cryopreserved using the freezing equilibration solutions and

cryopreservation solutions of Example 11 and Comparative Example 2 according to the schemes in Table 6 and Table 7. The freezing and thawing methods were the same as those in Application Example 1.

Table 6. Survival rates of cryopreserved mouse oocytes

No.	Equilibration solution	Cryopreservation solution	Thawing solution	Total number of frozen oocytes	Survival rates after 2 h
Application Embodiment 19	a	G	Thawing solution 1#	67	98.5%
Application Embodiment 20	a	H	Thawing solution 1#	109	96.3%
Application Embodiment 21	a	I	Thawing solution 1#	67	95.5%
Comparative Embodiment 10	a	Freezing solution 1#	Thawing solution 1#	146	95.0%

Table 7. Survival rates of cryopreserved mouse embryos

No.	Equilibration solution	Cryopreservation solution	Thawing solution	Total number of frozen embryos	Survival rates after 2 h
Application Embodiment 22	a	J	Thawing solution 1#	25	100.00 %
Comparative Embodiment 11	a	Freezing solution 1#	Thawing solution 1#	38	94.30%

As can be seen from the data in Tables 6 and 7, when the cryopreservation solution disclosed herein is used for cryopreservation of oocytes and embryos after the amount of DMSO and EG is reduced, the survival rate of the oocytes can reach no less than 95%, the survival rate of the embryos can reach 100%, a cryopreservation thawing rate of the cryopreservation solution disclosed herein can reach or even be far higher than that of a commercial cryopreservation solution comprising 15% DMSO and commonly available in clinical practice at present (in Comparative Embodiments 10–11), and the cryopreservation effect with the addition of the biomimetic amino acid ice growth inhibition material is remarkably superior to the cryopreservation effect without the addition of the biomimetic amino acid ice growth inhibition material.

[Application Example 5] Cryopreservation of human umbilical cord mesenchymal stem cells

Human umbilical cord mesenchymal stem cells were cryopreserved using the cryopreservation solutions of Example 11 and Comparative Example 2 according to the scheme in Table 8. Freezing and thawing methods are seen from Application Example 2.

Table 8. Survival rates of cryopreserved human umbilical cord mesenchymal stem cells

No.	Cryopreservation solution	Cryopreservation method	Survival rates
Application Embodiment 23	J	Microdroplet method	81.2%
Application Embodiment 24	K	Microdroplet method	82.6%
Application Embodiment 25	L	Microdroplet method	80.5%
Comparative Embodiment 12	Freezing solution 1#	Microdroplet method	63.9%
Comparative Embodiment 13	Freezing solution 3#	Microdroplet method	76.6%

When the cryopreservation solution disclosed herein is used for cryopreservation of human umbilical cord mesenchymal stem cells, the survival rate of the stem cells can reach no less than 80% by adding only a small amount of DMSO (7.5%) or even not adding DMSO (for example, in Application Embodiments 23–25). This means that the freezing reagent can not only have the same effectiveness in freezing stem cells as a conventional cryopreservation solution, but also has a cryopreservation thawing rate even far higher than that of a commonly available cryopreservation solution comprising 10% DMSO (in Comparative Embodiment 13), and the cryopreservation effect with the addition of the biomimetic amino acid ice growth inhibition material is remarkably superior to the cryopreservation effect without the addition of the biomimetic amino acid ice growth inhibition material (in Comparative Embodiments 14 and 15).

[Application Example 6] Cryopreservation of ovarian organs and ovarian tissues

The ovarian organs of mice newly born within 3 days and the ovarian tissue slices of sexually mature mice were cryopreserved using the freezing equilibration solutions and cryopreservation solutions of Example 11 and Comparative Example 2 according to the schemes in Table 9 and Table 10. Methods for freezing and thawing ovarian organs and ovarian tissues of sexually mature mice are seen from Application Example 3.

Table 9. Ovarian organ cryopreservation scheme

No.	Equilibration solution	Cryopreservation solution	Thawing solution	Morphology
Application Embodiment 26	a	J	Thawing solution 1#	FIG. 34
Application Embodiment 27	a	L	Thawing solution 1#	FIG. 35
Application Embodiment 28	a	K	Thawing solution 1#	FIG. 36
Comparative Embodiment 14	a	Freezing solution 1#	Thawing solution 1#	FIG. 25

Table 10. Ovarian tissue cryopreservation scheme

No.	Equilibration solution	Cryopreservation solution	Thawing solution	Morphology
Application Embodiment 29	a	J	Thawing solution 1#	FIG. 37
Application Embodiment 30	a	L	Thawing solution 1#	FIG. 38
Application Embodiment 31	a	K	Thawing solution 1#	FIG. 39
Comparative Embodiment 15	a	Freezing solution 1#	Thawing solution 1#	FIG. 30

[Example 11] Preparation of cryopreservation solution comprising peptidic compound as ice growth inhibition material

A cryopreservation solution M comprises the following components per 100 mL:

Substances	Content
TR (g)	28
DMSO (mL)	7.5
Ethylene glycol (mL)	10
Sucrose ($mol \cdot L^{-1}$)	0.5
Fetal bovine serum (mL)	20
DPBS (mL)	Balance

Solution preparation steps (total volume: 100 mL): 28 g of TR was ultrasonically dissolved in 25 mL of DPBS, and pH was adjusted to 7.0 to give a solution 1; 0.05 mol of sucrose was ultrasonically dissolved in 25 mL of DPBS, and after the sucrose was completely dissolved, 10 mL of ethylene glycol and 7.5 mL of DMSO were sequentially added to give a solution 2; after returning to room temperature, the solution 1 and the solution 2 were homogeneously mixed, the pH was adjusted, the volume was made up to 80% with DPBS, and finally 20 mL of serum was added before the cryopreservation solution was used.

A cryopreservation solution N comprises the following components per 100 mL:

Substances	Content
TPT (g)	28
DMSO (mL)	7.5
Ethylene glycol (mL)	10
Sucrose ($mol \cdot L^{-1}$)	0.5
Fetal bovine serum (mL)	20
DPBS (mL)	Balance

Solution preparation steps (total volume: 100 mL): 28 g of TPT was ultrasonically dissolved in 25 mL of DPBS, and pH was adjusted to 7.0 to give a solution 1; 0.05 mol of sucrose was ultrasonically dissolved in 25 mL of DPBS, and after the sucrose was completely dissolved, 10 mL of ethylene glycol and 7.5 mL of DMSO were sequentially added to give a solution 2; after returning to room temperature, the solution 1 and the solution 2 were homogeneously mixed, the pH was adjusted, the volume was made up to 80% with DPBS, and finally 20 mL of serum was

added before the cryopreservation solution was used.

A cryopreservation solution O comprises the following components per 100 mL:

Substances	Content
TR (g)	28
Ethylene glycol (mL)	10
Sucrose ($mol \cdot L^{-1}$)	0.5
Fetal bovine serum (mL)	20
DPBS (mL)	Balance

Solution preparation steps (total volume: 100 mL): 28 g of TR was ultrasonically dissolved in 25 mL of DPBS, and pH was adjusted to 7.0 to give a solution 1; 0.05 mol of sucrose was ultrasonically dissolved in 25 mL of DPBS, and after the sucrose was completely dissolved, 10 mL of ethylene glycol was added to give a solution 2; after returning to room temperature, the solution 1 and the solution 2 were homogeneously mixed, the pH was adjusted, the volume was made up to 80% with DPBS, and finally 20 mL of serum was added before the cryopreservation solution was used.

Preparation of freezing equilibration solutions: the freezing equilibration solutions were prepared according to the following formulations.

A freezing equilibration solution a: 7.5 mL of ethylene glycol and 7.5 mL of DMSO were added to 65 mL of DPBS, and mixed homogeneously, and 20 mL of serum was added when the freezing equilibration solution was used.

Comparative Example 3:

A freezing equilibration solution a comprises, per 1 mL, 7.5% (v/v) of DMSO, 7.5% (v/v) of ethylene glycol, 20% (v/v) of fetal bovine serum and the balance of DPBS;

A cryopreservation solution 1# comprises, per 1 mL, 15% (v/v) of DMSO, 15% (v/v) of ethylene glycol, 20% (v/v) of fetal bovine serum, 0.5 M sucrose and the balance of DPBS.

A cryopreservation solution 3# comprises, per 1 mL, 10% (v/v) of DMSO, 15% (v/v) of fetal bovine serum and the balance of a-MEM (USA, Invitrogen, C12571500BT).

The formulation of the thawing solutions used in Example 12 and Comparative Example 3 was as follows:

A thawing solution 1# comprises a thawing solution I (comprising sucrose at $1.0 mol \cdot L^{-1}$, 20% of serum and the balance of DPBS), a thawing solution II (comprising sucrose at $0.5 mol \cdot L^{-1}$, 20% of serum and the balance of DPBS), a thawing solution III (comprising sucrose at $0.25 mol \cdot L^{-1}$, 20% of serum and the balance of DPBS), and a thawing solution IV (comprising 20% of serum

and the balance of DPBS).

[Application Example 7] Cryopreservation of oocytes and embryos

Oocytes and embryos were cryopreserved using the freezing equilibration solutions and cryopreservation solutions of Example 13 and Comparative Example 2 according to the schemes in Table 11 and Table 12. The freezing and thawing methods were the same as those in Application Example 1.

Table 11. Survival rates of cryopreserved mouse oocytes

No.	Equilibration solution	Freezing solution	Thawing solution	Total number of frozen oocytes	Survival rates after 2 h
Application Embodiment 32	a	M	Thawing solution 1#	93	96.2%
Application Embodiment 33	a	N	Thawing solution 1#	48	90%
Comparative Embodiment 16	a	Freezing solution 1#	Thawing solution 1#	146	95%

Table 12. Survival rates of cryopreserved mouse embryos

No.	Equilibration solution	Freezing solution	Thawing solution	Total number of embryos	Survival rates after 2 h
Application Embodiment 34	a	M	Thawing solution 1#	41	95.9%
Comparative Embodiment 17	a	Freezing solution 1#	Thawing solution 1#	38	94.3%

The data in Tables 11 and 12 show that the polypeptides disclosed herein are used for cryopreservation of oocytes and embryos, and that the survival rates of oocytes and embryos of the existing commercial cryopreservation solution (DMSO content 15%) can be achieved by adding only a small amount of DMSO (7.5%), and the data of Application Embodiments 32 and 34 show that TR polypeptides have a more excellent cryopreservation effect on oocytes and embryos.

[Application Example 8] Cryopreservation of human umbilical cord mesenchymal stem cells

Human umbilical cord mesenchymal stem cells were cryopreserved using the cryopreservation solutions of Example 12 and Comparative Example 3 according to the scheme in Table 13. Freezing and thawing methods are seen from Application Example 2.

Table 13. Survival rates of cryopreserved human umbilical cord mesenchymal stem cells

No.	Cryopreservation solution	Cryopreservation method	Survival rates
Application Embodiment 35	M	Microdroplet method	87.8%
Application Embodiment 36	O	Microdroplet method	75.1%
Comparative Embodiment 18	Freezing solution 3#	Microdroplet method	76.6%

According to the results in Table 13, the cryopreservation solution disclosed herein without adding DMSO or adding only a small amount of DMSO (7.5%) can have a cell survival rate equivalent to that of a cryopreservation solution comprising 10% DMSO in the prior art, so that the amount of DMSO is greatly reduced, the damage and toxicity of DMSO to cells are reduced, and the passage stability and cell activity of the frozen stem cells can be greatly improved.

[Application Example 9] Cryopreservation of ovarian organs and ovarian tissues

The ovarian organs of mice newly born within 3 days and the ovarian tissue slices of sexually mature mice were cryopreserved using the freezing equilibration solutions and cryopreservation solutions of Example 12 and Comparative Example 3 according to the schemes in Table 14 and Table 15. Methods for freezing and thawing ovarian organs and ovarian tissues of sexually mature mice are seen from Application Example 3.

Table 14. Ovarian organ cryopreservation scheme

No.	Equilibration solution	Cryopreservation solution	Thawing solution	Morphology
Application Embodiment 37	a	M	Thawing solution 1#	FIG. 40
Comparative Embodiment 19	a	Freezing solution 1#	Thawing solution 1#	FIG. 25

Table 15. Ovarian tissue cryopreservation scheme

No.	Equilibration solution	Cryopreservation solution	Thawing solution	Morphology
Application Embodiment 38	a	M	Thawing solution 1#	FIG. 41
Comparative Embodiment 20	a	Freezing solution 1#	Thawing solution 1#	FIG. 30

As can be seen from FIGs. 24, 25 and 40, compared with the comparative embodiments free of the biomimetic peptide ice growth inhibition material (FIGs 25 and 30), a picture of a slice of the thawed ovarian organ cryopreserved in the cryopreservation solution of Application Embodiment 37 shows that the follicle structure is relatively intact, the interstitial structure is relatively intact, the cytoplasm is relatively homogeneous and lightly stained in a relatively large amount, and nucleus shrinkage and deep staining were relatively mild; and blood vessels have intact vessel wall structures and less luminal collapse, the cytoplasm of endothelial cells is relatively homogeneous and lightly stained in a relatively large amount, and nucleus shrinkage and deep staining were relatively mild. As can be seen, Application Embodiment 37 has a better cryopreservation effect on ovarian organs.

As can be seen from FIGs. 29, 30 and 41, compared with fresh unfrozen ovarian tissues of mature mice of Comparative Embodiment 22, the scheme of Application Embodiment 38 is characterized in that: the structures of follicles in the growth phase and antral follicles are relatively intact. It can be seen that the cryopreservation solution disclosed herein has a better cryopreservation effect on ovarian tissues than the prior art.

It can be seen that the cryopreservation solution prepared with the biomimetic peptide ice growth inhibition material as a main component disclosed herein can be simultaneously applied to cryopreservation of oocytes, embryos, stem cells, reproductive organs and tissues, where high cell survival rates and good biological activity can be achieved.

The examples of the present invention have been described above. However, the present invention is not limited to the above embodiments. Any modification, equivalent, improvement and the like made without departing from the spirit and principle of the present invention shall fall within the protection scope of the present invention.

CLAIMS

1. A method for screening an ice growth inhibition material, comprising: (1) determining the affinity of the ice growth inhibition material for water; and (2) determining the spreading performance of the ice growth inhibition material at an ice-water interface;
preferably, the step (1) is achieved by determining the solubility, the hydration constant, the dispersion size of the ice growth inhibition material in water, and/or the number of intermolecular hydrogen bonds formed between a molecule of the ice growth inhibition material and a water molecule.

2. The method for screening an ice growth inhibition material according to claim 1, wherein the step (2) is achieved by determining the amount of the ice growth inhibition material absorbed on an ice surface by an ice adsorption experiment,
the amount of the ice growth inhibition material absorbed on the ice surface = (the mass m_1 of the ice growth inhibition material adsorbed on the ice surface/the total mass m_2 of the ice growth inhibition material in an original solution comprising the ice growth inhibition material) × 100%.

3. The method for screening an ice growth inhibition material according to claim 1 or 2, wherein the ice adsorption experiment comprises:
S1, preparing an aqueous solution of the ice growth inhibition material, and cooling to a supercooling temperature;
S2, placing a pre-cooled temperature-regulating rod in the aqueous solution to induce an ice layer to grow on the surface of the temperature-regulating rod, continuously stirring the aqueous solution to enable the ice growth inhibition material to be gradually adsorbed onto the surface of the ice layer, and keeping the temperature of the temperature-regulating rod and the temperature of the aqueous solution at a supercooling temperature; and
S3, determining the amount of the ice growth inhibition material absorbed on the ice surface;
preferably, the temperature-regulating rod is pre-cooled in one of modes of freezing by liquid nitrogen, dry ice or an ultra-low temperature refrigerator;
preferably, the supercooling degree and the adsorption time are maintained unchanged during the ice adsorption experiment to ensure that the surface area of the resulting ice is maintained unchanged within an allowable error range;
preferably, wherein the ice growth inhibition material in the step S1 is fluorescently pre-labeled, for example, with fluorescein;

preferably, if the ice growth inhibition material itself has absorption characteristics in an ultraviolet or fluorescence spectrum, no fluorescent label is required;

preferably, wherein the step S3 comprises:

S3a, taking out an ice block after adsorption, rinsing the ice block with purified water, and melting the ice block to give an adsorption solution of the ice growth inhibition material; and

S3b, determining the volume V of the melted adsorption solution of the ice growth inhibition material, determining the mass/volume concentration c of the ice growth inhibition material in the adsorption solution, and calculating the mass m_1 of the ice growth inhibition material adsorbed on the ice surface through the formula $m_1 = cV$;

preferably, wherein in the S3b, the concentration c is determined by ultraviolet-visible spectroscopy;

preferably, wherein the method is used for screening the material for inhibiting the growth of ice crystals.

4. The method for screening an ice growth inhibition material according to any one of claims 1–3, further comprising a step (3): evaluating the affinity of the material for water and the affinity of the material for ice, wherein the material with strong affinities for water and ice has good ice growth inhibition performance.

5. An ice adsorption experimental device for use in the method according to any one of claims 1-4, comprising a multilayer liquid storage cavity, a temperature-regulating rod and a temperature regulator, wherein the multilayer liquid storage cavity sequentially comprises an ice adsorption cavity, a bath cavity and a cooling liquid storage cavity from inside to outside, the temperature-regulating rod being arranged in the ice adsorption cavity, and the temperatures of the temperature-regulating rod and the liquid storage cavity being regulated by the temperature regulator;

preferably, the temperature-regulating rod is of a hollow structure made of a thermally conductive material, and the hollow structure of the temperature-regulating rod is provided with a liquid inlet and a liquid outlet; the temperature regulator is a fluid temperature regulator and is provided with a cooling liquid outflow end and a reflux end; two ends of the cooling liquid storage cavity is provided with a liquid inlet and a liquid outlet; the cooling liquid outflow end of the temperature regulator, the liquid inlet of the temperature-regulating rod, the liquid outlet of the temperature-regulating rod, the liquid inlet of a cooling liquid storage tank, the liquid outlet of the cooling liquid storage tank and the reflux end of the temperature regulator are

sequentially linked via pipelines through which a cooling liquid flows;

preferably, the multilayer liquid storage cavity is provided with a cover;

preferably, when the ice adsorption experimental device is used, the ice adsorption cavity is arranged to contain the aqueous solution of the ice growth inhibition material, and the bath cavity in the middle layer is arranged to contain a bath medium that is at a preset temperature, for example, a water bath, an ice bath or an oil bath; after the preset temperature of the cooling liquid is reached, the cooling liquid flows out through the temperature regulator and flows into the hollow structure of the temperature-regulating rod to regulate the temperature of the temperature-regulating rod, then flows out from the liquid outlet of the temperature-regulating rod and flows into the cooling liquid storage cavity in the outer layer to maintain the temperature of the bath medium at the preset level, and then flows through the liquid outlet of the cooling liquid storage tank and the reflux end of the temperature regulator and enters the temperature regulator to circulate.