Chapter

The Use of Chitooligosaccharides in Cryopreservation: Discussion of Concept and First Answers from DSC Thermal Analysis

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Abstract

The use of dimethyl sulfoxide (Me_2SO) as a cryoprotectant agent (CPA) is controversial. Indeed, this cryoprotectant agent (CPA) is cytotoxic and potentially mutagenic. Therefore, other cryoprotectants must be used to reduce the proportion of Me_2SO in slow-freezing solutions. In this chapter, we propose to present the first evaluation of new non-penetrating cryoprotectants: the chitooligosaccharides (COS). These molecules are chitosan oligomers, which are biocompatible, antioxidant, and bacteriostatic. We first review the use of saccharides through cryopreservation processes. We question the possibility to reduce penetrating CPA during slow-freezing procedures. We propose to use COS as extracellular CPA to reduce the use of Me_2SO. We question the biocompatibility of COS on mouse embryos through the analysis of the cells' development. Next, we evaluate these molecules in slow-freezing solutions with a reduced quantity of Me_2SO. Our experimental approach is a physical method often used to characterize slow-freezing solutions. Differential scanning calorimetry (DSC) allows to evaluate the crystallization and melting processes, the amount of crystallized water, and the equilibrium temperature and consequently to evaluate the impact of different cryoprotectants. This study gives a better understanding on how slow-freezing protocols could be improved with extracellular CPA.

Keywords: slow-freezing improvement, chitooligosaccharides, dimethyl sulfoxide, physical approach, differential scanning calorimetry

1. Introduction

Despite the progress achieved these last decades, the improvement of cryopreservation procedures is still desired by the scientific community [1, 2]. A possible improvement is the decrease of the penetrating cryoprotective agents (CPA). The small and osmotically active penetrating molecules used as penetrating CPA in cryopreservation present risks of cytotoxicity for cells [3–6]. These molecules penetrate easily into tissues and, after impregnation, are difficult to fully extract from

biological systems. Among the different penetrating CPA, Me_2SO is a penetrating CPA especially efficient to promote successful cryopreservation. However, the Me_2SO molecule presents risks of toxicity to biological materials [7–14]. It is also suspected to be an intercalator of DNA [15]. In this chapter, we propose to study the possibility to reduce the use of the Me_2SO molecule while maintaining the survival rate of cells in slow-freezing cryopreservation.

A challenge of modern cell cryopreservation is to propose procedures in which CPA concentration is as little as possible:

- In vitrification procedures, the techniques seek to obtain the glassy state with very small amounts of penetrating CPA (less than 25% (w/w)) by replacing them with non-penetrating CPA [3, 16–18]. Accordingly, the cooling must be applied as quickly as possible so that the cells do not suffer the damage associated with excessive dehydration and/or volume loss at room temperature. They also require the use of particularly small sample volumes (<1 μL) to reach high cooling rate that will prevent ice crystallization. These procedures are particularly delicate and require a certain dexterity of the experimenter [19, 20].

- In slow-freezing procedures, reducing the initial amount of penetrating CPA significantly increases the stresses and risks experienced by cells during cryopreservation. It is therefore difficult to propose an excessive reduction in the initial concentration of penetrating CPA. Indeed, it has been theoretically demonstrated, through mathematical modeling, that it is impossible to cryopreserve cells with initial Me_2SO concentrations below 1 $mol.L^{-1}$ [21].

We assume, however, that a good combination of penetrating and non-penetrating CPA and an adaptation of the slow-freezing protocols could provide satisfactory survival rates, despite a reduction in the initial proportion of Me_2SO used. This chapter investigates an alternative to the current slow-freezing procedures using Me_2SO and proposes procedures where the initial and necessary amount of Me_2SO is lowered.

Decreasing the Me_2SO proportion in solution will modify the couple "protocol/solution" within the cryopreservation procedure since it is necessary to counterbalance the loss of the cryoprotective effect of the removed Me_2SO molecules. To that end, this chapter studies the possibility to use chitooligosaccharide (COS) compounds in solution. The role of extracellular CPA is discussed, and the effect of the COS in solution is thermodynamically evaluated.

Even if common characteristics of various CPA can be defined, the substances currently used as CPA have different chemical structures and sizes, so it is still difficult to predict the cryoprotective properties of substances from their basic chemical structures. The modes of action of cryoprotectants have not yet been fully elucidated. Thus, the development of effective cryopreservation solutions remains primarily based on empirical considerations. Consequently, studying the potential cryoprotective characteristics of new compounds serves a dual purpose: (1) propose new cryoprotectants and (2) understand the mechanisms of cryoprotection. It makes possible to better classify the molecules according to their modes of action regarding cellular cryoprotection.

This chapter aims to assess the use of COS to fulfill part of the cryoprotective role of the Me_2SO. It presents first, from the cellular cryopreservation point of view, the interesting properties of saccharides (Part 2). It discusses next the optimization of the procedures required to replace a part of the penetrating CPA by using non-penetrating saccharides (Part 3). The use of COS to realize this optimization is

explained (Part 4). Then, COS synthesis is presented from a physicochemical point of view (Part 5). The biocompatibility of COS on mouse embryos is questioned (Part 6), and a first understanding of the action of COS as cryoprotectants is quantified by DSC analysis (Part 7), before a conclusion on the possibility of using these compounds as non-penetrating CPA.

2. State of the art: the use of saccharides in cryopreservation

The cryoprotective role of saccharide compounds (mono-, di-, tri-, oligo-, or polysaccharides) is known for a long time. Some of them are secreted by certain cold-blooded organisms that withstand harsh winters [22, 23]. Several saccharides are water soluble, have a high osmotic effect, and have many influences on the solutions' properties: they can bind part of the water and make it non-crystallizable [24–29]; their action on viscosity [30, 31] has been highlighted, as well as their ability to disrupt the organization of water molecules in the liquid phase [32–35]. In a mixture of "H_2O/saccharide compound," the proportion of water has an influence on the properties of the saccharide compound and therefore on the properties of the mixture [29, 36].

These compounds are known to be unable to penetrate passively through most cell membranes. From the cryoprotective point of view, they act outside the cells, giving them limited action on cell protection. This is the reason why the standard in cryopreservation procedures remains the massive use of penetrating CPA that possess a high osmotic effect in both extra- and intracellular solutions. However, the beneficial use of saccharides to substitute penetrating CPA compounds has been proven in recent years. Indeed, saccharides have been used for cell cryopreservation in slow-freezing [37–51] or vitrification procedures [16–18, 39, 52–55]. A non-exhaustive overview from the literature of the use of the saccharides is displayed in **Table 1**.

Using empirical methods, it has been shown that these compounds allow the improvement of the post-cryopreservation survival rates of certain cell types [3, 37, 38, 44–48, 82]. Some authors have discussed the benefits of using them:

- Meryman was the first to propose that the presence of large polymers in solutions prevents the risks of osmotic shocks (when the penetrating CPA are released from the intracellular medium) as well as the denaturation of cell membranes [83].

- In cryopreservation of spermatozoa, small saccharides are often used as substituents for penetrating CPA [42, 49, 50, 61]. The advanced argument of their use is that they increase the viscosity, increase the glass-forming tendency, and protect the cellular lipid membranes [42, 61, 82].

Monosaccharides	**Disaccharides**	**Trisaccharide**
Glucose [56–63]	Sucrose [61, 64–67]	Raffinose [14]
Fructose [14]	Maltose [35]	**Polysaccharides**
Sorbitol [14]	Trehalose [16, 35, 39–43, 49–51, 61, 68–79]	Dextran [1, 2, 17, 18, 54, 67, 80, 81]
Mannitol [14]		
Galactose [64]		Ficoll [1, 17, 18, 54, 64, 66, 81]

Table 1.
Non-exhaustive directory from literature of saccharides used in cryopreservation procedures.

- In cryopreservation by vitrification, Kuleshova et al. [17] proposed to replace a large portion of penetrating CPA by large extracellular polysaccharides (Ficoll and dextran).

- In slow-freezing cryopreservation, saccharides have also been used [84]. For the cryopreservation of human or mouse embryos, Dumoulin et al. [81] highlighted the ability of large molecules (e.g., dextran) to reduce damage to zona pellucida when added in small amounts (i.e., less than 10% (w/v)). For the cryopreservation of cat embryos, Gómez et al. [67] were able to obtain interesting survival rates (>80%) by combining propylene glycol (1.4 M), sucrose (0.125 M), and dextran 10% (w/w). Slow-freezing procedures for some weak cellular systems have been proposed, without recourse to CPA penetrants [43]. This strategy is based on the induction, prior to ice formation, of cellular dehydration using osmotically active extracellular compounds (e.g., trehalose).

- For the slow-freezing cryopreservation of human red blood cells, Bailey et al. [85] reported the possibility to reduce the use of Me_2SO from 10% (w/w) to 2.5% (w/w) through the use of high concentration of copolymers (polyampholytes). These authors concluded that because no ice recrystallization inhibition (IRI) activity could be seen, the interaction of those copolymers with cells' membranes should be the reason that increases the cell recovery.

From these readings, mechanisms can be advanced to explain how the addition of saccharides in solution improves cell survival:

- Saccharides have a cryoscopic influence in the extracellular medium [86]. It reduces the damage associated with the formation and growth of the crystalline phase.

- Saccharides promote cell dehydration on cooling [17]. This aspect is ambivalent because, on the one hand, it allows to promote intracellular vitrification, but on the other hand, it increases the risks associated with cellular dehydration.

- Saccharides reduce the cellular rehydration during warming [19, 83]. By having an osmotic effect from the extracellular medium, they limit the osmotic gap (appeared during cooling, due to the difference between the intra- and the extracellular vitrification temperature [87]) between intra- and extracellular media. During warming and during the ice melting, saccharides guarantee a smoother return of water into the cells [19, 83].

- Saccharides participate in the evolution of extracellular viscosity. This reduces the kinetics of crystal growth and favors the deviation of the system from its equilibrium position. This effect has the consequence of improving the extracellular vitreous state achievement [17].

- Saccharides allow to "encapsulate" cells. Thus, the presence of large molecules in solution protects the zona pellucida of embryos during slow-freezing procedures [81]. Likewise, the presence of these large molecules can move the cells away from the ice in the overconcentrated amorphous phase [82]. It has been also reported that the presence of mono- or disaccharides in solution

improves sperm motility after cryopreservation [88]. Finally, it has been proposed to create hydrogel-based microspheres to encapsulate cellular systems in small volumes [89].

- Saccharides promote the lipid transition of phospholipids from cell membranes. By interacting with cell membranes, saccharides have an effect on the fluidity of lipidic membranes [82, 83]. Interactions between membrane phospholipids and saccharides in solution have also been advanced as a method of promoting the success of cryopreservation procedures [90, 91].

Saccharidic CPA are used in different ways depending on their physicochemical characteristics. In general, the polysaccharides are added in solution to increase the viscosity while having a weak osmotic activity. On the contrary, the small saccharide molecules (such as glucose) are added in solution to have high osmotic activity within the solution (this favors and induces the cellular dehydration), without changing the viscosity too much.

3. Strategy for improving slow-freezing cryopreservation procedures

3.1 Foundations of the strategy

Models showed that the improvement of cryopreservation procedures is impossible if it implies an excessive reduction in the use of penetrating CPA (<1 mol.L^{-1}) [21]. But these models studied this reduction without trying to use other CPA compounds with completely different properties than those currently used. Me_2SO is certainly one of the best compounds to fulfill the roles that are required to a CPA (apart from its cytotoxicity), but it is conceivable that a combination of different CPA may also be compatible with cell survival.

To reduce the use of Me_2SO in slow-freezing cryopreservation procedures, several assumptions were considered, based on results published in the literature:

a. The slow-freezing procedures have been optimized according to some parameters, but new optimizations are possible.

Other combinations of the parameters may be better than the currently used. The search for new CPA is still ongoing, and the cryopreservation procedures are better understood. The discovery of intracellular vitreous transitions [87], the control of the IRI process [92], the development of new cooling or warming techniques [93], etc. may suggest that optimization of the procedure is still possible.

b. The optimization of the proportion of penetrating CPA in the cryopreservation solution is considered in order to find a formulation offering good vitrification conditions for the remaining solution.

It has been shown that the proportion of penetrating CPA necessary to lead to a cellular vitrification can be reduced if there is a high cellular dehydration [16]. Indeed, the intracellular cytoplasmic medium has a great glass-forming tendency because of the presence of many macromolecules [87, 94–96]. The presence in intracellular solution of a large proportion of proteins and other organic compounds (macromolecules), accompanied by dehydration of the medium, is sufficient to promote vitrification [87, 94]. In addition, experiments have shown the existence of colloidal glass transitions in cells during their dehydration [96, 97]. These transitions can promote intracellular vitrification and potentially protect cells (since

cell cytoplasm is often heavily loaded with large molecules, it can easily vitrify [17, 59, 96]). In a vitrification procedure, using successive baths of osmotic equilibration, the cells are placed in solutions containing penetrating CPA. It was proposed by Kuleshova et al. [17] to replace, in those procedures, a large proportion of penetrating CPA with non-penetrating polymers (PVP, dextran, Ficoll, etc.). In the last osmotic equilibration bath, non-penetrating CPA (sugars or polymers) are added to promote cell dehydration just before the plunge into liquid nitrogen (LN2). The osmotic effect of these non-penetrating CPA promotes the vitrification of the intracellular medium and ensures the success of the procedure with a lower amount of penetrating CPA [16]. By adaptation to slow-freezing procedures, we assume that it is possible to force the intracellular colloidal glass transition to the correct temperature by suitably combining temperature lowering and cellular dehydration. However, cellular dehydration is not possible over long periods when the temperature remains high. During a vitrification procedure, the dehydration time is short (primarily applied during the last osmotic bath, a few seconds before the plunge into LN2). During slow-freezing, if the dehydration intervenes too extremely, it could lead to cellular collapse [83, 98, 99]. To solve this problem, we rely on the work of Mazur et al. [100] who proposed an alternative to conventional cellular cryopreservation protocols by rapidly bringing the cells to a temperature low enough to make the effects of cell dehydration less deleterious. This temperature must, however, remain above the nucleation temperature of the intracellular medium. A temperature stabilization is then carried out at this intermediate temperature to allow the equilibration of the media. Then, the cooling continues at a slow cooling rate until the plunge into LN2. This type of protocol is called “rapid cooling interrupted” [100]. It should be noted that this protocol also possesses a seeding step in order to control the extracellular ice growth. We assume that, when using this type of protocol, it would be possible to allow faster cell dehydration already biologically tolerated in the case of vitrification procedures.

c. The use of non-penetrating CPA can allow reducing the necessary proportion of penetrating CPA by optimizing the procedure of cooling and warming.

For a slow-freezing procedure, in which ice formation is allowed and desired, the osmotic effect of a non-penetrating CPA becomes increasingly important as the proportion of water in the remaining extracellular medium decreases. Conversely, in the intracellular medium, it is mainly the penetrating CPA that participates in the osmotic balance. There is therefore a gap between these two media in the osmolality evolution, which accentuates cellular dehydration and increases cell contraction at a given cooling rate. The use of an osmotically active non-penetrating CPA in solution may induce a significant change in the osmolality difference between the intra- and the extracellular media.

3.2 The elements of the proposed optimization

The toxicity of the cryopreservation solutions is related to the CPA concentration reached in the remaining solution to enable the vitrification of the intracellular medium. Moreover, to protect the cells, the vitrification must also be carried out in the noncrystalline extracellular medium in contact with the cells. Intracellular vitrification is dependent on the presence of penetrating CPA and on the cellular dehydration. In consequence, the intracellular vitrification is dependent on the diffusion of materials across the cellular membrane. That is why cryopreservation procedures are time dependent. Indeed, an ideal cooling rate exists at which, for a specific cell (i.e., a specific permeability and cell size), the cellular dehydration is ideally compensated by

the CPA penetration. This rate deals with the two major risks of the slow-freezing procedures, the "solute effect," which occurs at low cooling rate, and the intracellular nucleation, which occurs at high cooling rate [101].

The addition of penetrating CPA in a solution, in a reasonable proportion (> 1 mol. L^{-1} for slow-freezing and > 3 mol.L^{-1} for vitrification), is considered essential for the survival of mammalian embryo type cells [21]. Very few studies have been published on attempts to reduce the amount of Me_2SO in solution during slow-freezing cryopreservation [43, 84]. This strategy was evaluated by relying on modeling arguments, showing that it seems complicated to reduce this initial proportion of penetrating CPA [21]. We make a proposal for slow-freezing procedure optimization using a reduced initial proportion of penetrating CPA, supplemented with non-penetrating CPA.

The decrease of the initial proportion of penetrating CPA implies an increase in the amount of crystallizable water, a stronger evolution of the crystal/liquid ratio in the solution with temperature, a higher amount of penetrating CPA needed to diffuse through the membrane, more risks of contact between cells and ice crystals, etc. Thus, penetrating CPA play a cryoprotective role that cannot be fully reproduced in the extracellular environment by non-penetrating CPA. Moreover, the stresses and risks associated with the ice formation must be limited or prevented until the plunge to LN_2. It is therefore necessary to continue to use penetrating CPA, but their amount in the initial solution should be limited to reduce cytotoxicity. In addition to the presence of penetrating CPA, the vitrification of the extracellular remaining solution depends on the presence of non-penetrating CPA that limit the evolution of penetrating CPA concentrations and reduce the risks associated with the ice formation in solution. The reduction of penetrating CPA implies to control the damages related to the ice formation in solution by replacing a large part of the initial extracellular water by non-crystallizable substances, which offer favorable conditions for cryopreservation. However, the biocompatibility of these compounds on the cellular systems must be guaranteed.

Because the proportion of CPA in the intracellular medium has a lighter impact than the cellular dehydration on the intracellular vitrification [16, 87, 94–96], we assume that the proportion of penetrating CPA may be limited. In that case, it is then necessary to reduce the deleterious effects related to the cellular dehydration by using non-penetrating CPA that are not highly osmotically active and whose molar concentrations slightly change during cooling (i.e., compounds with a large molar mass). It is also necessary to employ the "fast interrupt protocol" by suitably combining dehydration and lowering temperature so that the colloidal intracellular transition occurs before cell dehydration becomes too important.

Finally, it is necessary to verify that the extracellular remaining solution can vitrify easily so that the cryopreservation procedure can be interrupted at a higher plunge temperature into LN_2. Thus, the use of cryostabilizer CPA that facilitate the achievement of the vitreous state, without binding a significant amount of water, may promote the stabilization of the system "ice/remaining solution" (by reducing the risk of recrystallization of the intercrystalline remaining solution).

We chose to study the possibility to cryopreserve mouse embryos with 5% (v/v) of Me_2SO in the initial solution, supplemented with non-penetrating CPA (COS).

4. The proposition to use COS

The idea of using COS compounds in cell cryopreservation is supported by:

- An absence of toxicity, an ability to degrade without toxic residues, and a biocompatibility [102].

- Bacteriological, fungistatic, and antitumor properties [102–106].
- An ability to be soluble in aqueous solutions at physiological pH [107].
- A tendency to form aggregates in aqueous media [108].
- A chemical structure of oligosaccharide type close to molecules currently used in cryobiology.
- Potential interactions with water and consequently a potential effect on the properties of aqueous solutions (like that proposed by mono-, di-, and oligosaccharides).

In addition, because of the length of their chains, the COS propose intermediate properties between the polymers and the mono- or disaccharides. These properties are adjustable since the length of the chains can be selected during their synthesis using degree of polymerization (DP) parameter as well as the nature of the monomers present on the chain using the degree of acetylation (DA) parameter. These modular characteristics let us hope for the attainment of physicochemical properties that are adjustable for use as a CPA. Furthermore, they are particularly biocompatible and nontoxic. As a result, addition of COS to some penetrating CPA appears to be a relevant choice to reduce the needed amount of CPA during slow-freezing.

Assuming that a use of COS would reduce the use of Me_2SO, this work proposes to look for a composition of COS-based solution that can reproduce, in the extracellular medium, some effects of Me_2SO (favor the vitrification of the intracellular medium, promote the vitrification of the intercrystalline remaining solution, reduce the cell dehydration, etc.).

Our current procedure used for mouse embryo cryopreservation has been optimized for the "IMV" holding medium (embryo-holding medium, IMV® Technologies, L'Aigle, France) supplemented with approximately 10% (v/v) Me_2SO [109]. We set the goal of reducing by 50% the proportion of Me_2SO, to successfully cryopreserve mouse embryos using an IMV + 5% (v/v) Me_2SO solution containing a certain amount of COS.

5. Synthesis and chemical characterization of COS

The basic compound used to synthesize COS is chitosan supplied by the Indian company Mahtani Chitosan®. This chitosan (batch 244/020208) is produced from chitin extracted from shrimp shells. The chitosan provided was almost completely deacetylated (DA < 0.5%) by deacetylation reaction. This chitosan has a number-average molecular weight of 115 $kg.mol^{-1}$ and dispersity of 2.3. For the preparation of COS, the macromolecular chitosan chains are depolymerized according to a nitrous acid deamination reaction using sodium nitrite ($NaNO_2$) in acidic conditions [110]. It is only possible to obtain a statistical average chain length distribution around a mean DP value. To control the DA parameter, it is possible to perform an N-acetylation reaction of the D-glucosamine units with acetic anhydride as acetylation agent. In this chapter, DP and DA parameters have been used to name the COS as follows: COSDP_DA, with DP = "the average number of monomers per COS chain" and DA = "the average degree of acetylation of the chains of this COS."

To estimate the residual hydration rate in purified COS, we used data from the literature. A previous study of the same types of chitosan chains, but in a polymeric form, evaluated these hydration levels [111]. This study performed by

thermogravimetric analysis has shown that there are residual hydration levels after synthesis, purification, and lyophilization, which are variable according to the DA parameter of the chitosan polymers chains. From this data, we extrapolated the hydration levels of the COS compounds according to the DA parameter. For a DA ≈ 0%, we considered a residual hydration value of the COS compound equal to 6.5% (w/w). This is consistent with other results published in the literature where a value of 6% (w/w) was obtained [112].

6. Questioning the biocompatibility of COS

Before considering the use of COS in cryopreservation solutions, an evaluation of its biocompatibility is necessary. It ensures that their presence will not be deleterious to the future survival and development of the cryopreserved biological system. By their nature and chemical composition, the biocompatibility of COS has already been emphasized [113]. However, the molecular interactions between all the compounds in extracellular solution and the cell membranes are complex. There are, for example, interactions between COS molecules and cell membranes that are invoked to explain their fungistatic and bacteriostatic properties [113]. It is therefore not excluded that interactions may exist with eukaryotic cells. In addition, the cell system chosen for this study, the mouse embryo, is a fragile system whose development can be disrupted by the presence of harmful materials. Consequently, deleterious influence on this cellular system, linked to COS, must be excluded.

6.1 Materials and methods

The biocompatibility of COS was evaluated with mouse embryos. A procedure identical to that presented in a previous team article [109] was applied for the production of embryos used in this study. To study the biological action of COS on mouse embryos, a large quantity of COS10_0 (150 $mg.mL^{-1}$) was dissolved in the cryopreservation solution (IMV + 5% (v/v) Me_2SO) and in the "IMV" holding medium. One hundred twenty-six embryos (morula stage) were collected and were then mixed and divided into five groups (cf. **Table 2**): (1) "IMV," a control group placed in a solution without CPA; (2) "IMV + 10% (v/v) Me_2SO," a control group placed in the solution conventionally used for cryopreservation of mouse embryos; (3) "IMV + 5% (v/v) Me_2SO," a control group placed in the solution in which COS

	Groups	pH	Number of embryos in culture	Young blastocysts		Expanded blastocyst		Hatching	
1	IMV	7.29	25	23	92%	23	92%	23	92%
2	IMV + 10% (v/v) Me_2SO	7.29	25	20	80%	18	72%	14	56%
3	IMV + 5% (v/v) Me_2SO	7.30	25	23	92%	20	80%	20	80%
4	IMV + 5% (v/v) Me_2SO + (150 $mg.mL^{-1}$) COS_{10_0}	7.32	25	25	100%	22	88%	20	80%
5	IMV + (150 $mg.mL^{-1}$) COS_{10_0}	7.34	26	24	92%	23	88%	23	88%

The stage reached by the embryos was evaluated under a binocular microscope every 24 hours for 4 days. For each embryonic stage, the number of embryos observed at this stage is indicated in the left column and, on the right, the corresponding percentage in relation to the initial number of cultured embryos.

Table 2.
Compilation of embryonic developments observed by groups.

are diluted; (4) "IMV + 5% (v/v) Me_2SO + (150 mg.mL^{-1}) COS_{10_0}," a test group placed in a tested cryopreservation solution with COS; and (5) "IMV + (150 mg.mL^{-1}) COS_{10_0}," a second test group, without Me_2SO, studied to highlight the effect of COS and avoid the potentially cross-cutting effects between COS and Me_2SO.

The embryos were placed in these solutions at room temperature for 10 minutes. They were rinsed with M16 culture medium (IMV® Technologies, L'Aigle, France) and then placed in a culture chamber. The culture medium was equilibrated in the incubator (+37°C; 5% (v/v) CO_2; humid atmosphere), and then the embryos were introduced therein. The culture was maintained and supervised for 4 days. The development of embryos was then compared (every 24 hours) with other control groups to assess the state of the embryonic stage reached by each embryo. These comparisons can highlight certain harmful effects related to the presence of the different products on cellular development.

6.2 Results and discussions

After 24 hours, 20% of the embryos in the 10% (v/v) Me_2SO group failed to reach the "young blastocyst" stage. For groups with 5% (v/v) Me_2SO, the development stage remains similar to the solution without Me_2SO. The presence of COS does not appear to have a deleterious effect on the development of embryos at this stage, and the reduction in the proportion of Me_2SO in solution seems to be beneficial.

After 48 hours, it is possible to compare the number of embryos which reached the "expanded blastocyst" stage to the number of embryos which previously reached the "young blastocyst" stage. The numbers are the same for group 1 (control without CPA and COS), while an embryo did not develop in group 5 (with COS but without CPA), and more than one embryo did not develop in the other groups (2, 3, and 4). By comparing the results for groups 3 and 4, the effect of COS presence on embryo development may be considered minimal. Conversely, the decrease in the number of living embryos in groups 2, 3, and 4 seems to be directly associated with the presence of Me_2SO in the solution where embryos are bathed at room temperature.

After 72 hours, the number of embryos that reached the hatching stage is the least important for group 2 (with 10% (v/v) Me_2SO). There is no difference according to the presence or absence of COS in the IMV, and the final percentage in each case is very close. It is the same in solutions with 5% (v/v) Me_2SO, where the number of embryos which reached this stage of development is equivalent, with or without COS.

According to this study repeated only once, a negative effect of Me_2SO on the development of mouse embryos is highlighted. However, a negative effect of COS on this cellular development is discarded. These results should be repeated but seem to demonstrate the biocompatibility of COS for the mouse embryos. We conclude that COS can be used in the cryopreservation solutions in contact with cells.

7. Thermodynamic characterization of cryopreservation solutions containing COS

The strategy outlined in Part 3 aims to reduce by 50% the initial volume proportion of Me_2SO in slow-freezing solutions with the help of COS. It is thus necessary to know more precisely their mode of action in aqueous solutions. Several studies have already proposed the thermodynamic characterization of chitosan or chitosan derivatives [26, 112, 114–116]. However, these studies have focused on the

thermodynamic characterization of polymer chains. To our knowledge, the characterization of the thermodynamic properties of aqueous solutions formulated with soluble oligosaccharide derivatives of chitin or chitosan has not yet been done.

The objective of this study is to evaluate the impact of COS on the thermodynamic properties of aqueous solutions and to put forward a potential action of these products on water and other properties of the solutions (viscosity, gelling, etc.). It will help to evaluate the cryoprotection effect of COS.

7.1 Materials and methods

7.1.1 Thermodynamic characterization of solutions

A differential scanning calorimeter (DSC Diamond; Perkin Elmer) with power compensation was used in this study. The previously described procedures [117] have been applied, with the same methods of sample preparation, calibration of the DSC calorimeter, and precautions for use. COS were added in the IMV + 5% (v/v) Me_2SO solution, with different mass concentrations ($mg.mL^{-1}$) of hydrated powder of COS (per unit volume of the IMV + 5% (v/v) Me_2SO solution), as listed in **Table 3**.

To study the cryoprotective capability of COS as a substitute for Me_2SO, the solutions were studied by DSC using a protocol described elsewhere [117]. This protocol starts with a cooling from +10°C to −150°C at $-100°C.min^{-1}$, a warming from −150°C to +10°C at $+2.5°C.min^{-1}$, a cooling from +10°C to −150°C at $-2.5°C.min^{-1}$, and a warming from −150°C to +20°C at $+20°C.min^{-1}$. The experiments were repeated three times (n = 3) for each concentration.

7.1.2 Correction of the studied mass concentrations

The studied COS have a residual moisture content. Thus, the mass concentration of the added powder is not equivalent to the real mass concentration of the added product. Correction is made according to Eq. (1):

$$\textit{Real mass concentration of COS} = \textit{Powder mass concentration of hydrated COS} * \left(1 - \Psi_{H_2O,\,ini}(\mathrm{COS})\right) \quad (1)$$

Real mass concentration of COS ($mg.mL^{-1}$), powder mass concentration of hydrated COS ($mg.mL^{-1}$), $\Psi_{H_2O,\,ini}(\mathrm{COS})$, residual hydration ratio in the lyophilized powder of purified COS (Ø)

Products	Averaged DP	Averaged DA (%)	Residual hydration ratio after purification % (w/w)	Powder mass concentrations studied ($mg.mL^{-1}$)	Real mass concentrations studied ($mg.mL^{-1}$)	DSC sample mass (mg)
$COS_{7.5\_0}$	7.5	0	6.5	30; 60; 90; 150	28.05; 56.1; 84.15; 140.3	~5
COS_{10_0}	10	0	6.5	0; 25; 50; 100; 150; 200	0; 23.38; 46.75; 93.5; 140.3; 187	~10

Table 3.
Table of the studied products, with their characteristics (DP, DA, and residual hydration ratio after purification) and their studied mass concentrations.

The actual mass concentrations of COS in the solutions that were studied are presented in **Table 3**. In the following analyses, only the actual mass concentration of COS is considered for a given compound. However, to simplify the notation in thermograms, the hydrated powdered mass concentration of COS is used to designate the solutions.

7.1.3 Normalization of the transition enthalpies to 0°C

The amounts of ice formed in solution were estimated using the determination of the crystallization enthalpy (ΔH_c) and the melting enthalpy (ΔH_m). To estimate these amounts, the peak areas were calculated using a sigmoidal curve baseline (with the Pyris software 11.1.1). During warming the tangents, allowing the calculation of the sigmoidal line, were positioning, respectively, just before the colloidal transition and just after the melting peak.

In order to compare the transition enthalpies, a normalization of the measured values to a specified temperature of 0°C was made. As the enthalpy value of the transition of water to ice evolves as a function of temperature, the "latent heat of solidification of supercooled water" (Lf) was obtained at the measured phase change temperature from Boutron's [118] data interpolation. His data were calculated [119] from Angell's [120] specific heat capacity (Cp) of supercooled water and from Weast's Hand Book's [121] Cp of ice at different temperatures.

The normalization of the measured values to the expected values at the specified temperature of 0°C considers that the same proportion of solution transits at the transition temperature (T_t) ($\Delta H_{meas}(T_t)/L_f(T_t)$) and at 0°C ($\Delta H_{normalized}(0°C)/L_f(0°C)$). This equality leads to Eq. (2):

$$\Delta H_{normalized}(0°C) = \frac{\Delta H_{meas}(T_t) * L_f(0°C)}{L_f(T_t)} \quad (2)$$

where $\Delta H_{normalized}(0°C)$ is the transition enthalpy of the sample normalized to 0°C ($J.g^{-1}$), $\Delta H_{meas}(T_t)$ is the transition enthalpy of the sample measured at a transition temperature T_t ($J.g^{-1}$), $L_f(T_t)$ is the transition enthalpy of a pure water sample measured at a transition temperature T_t ($J.g^{-1}$), and $L_f(0°C)$ is the transition enthalpy of a pure water sample measured at 0°C (= 333.4 $J.g^{-1}$) ($J.g^{-1}$).

During cooling, to analyze the crystallization enthalpies, the nucleation temperature (T_n) was considered as the transition temperature (i.e., $T_t = T_n$). During warming, to analyze the melting enthalpies, the temperature of the summit of the melting peak (T_{max}) was considered as the transition temperature (i.e., $T_t = T_{max}$). These are approximations because for a mixture there is not only one transition temperature.

7.1.4 Comparison of measured and calculated transition enthalpies with the addition of an inert mass

When a non-hydrated inert mass is added in a solution, a lowering of the enthalpies of crystallization and melting is expected since this addition decreases, in proportion, the mass of water present in solution. The term "inert" refers to a product that does not influence the transition enthalpies (i.e., a product that does not transit and does not influence the transition of other products). The theoretical link between the decrease of the transition enthalpies and the decrease of the mass proportion of water in solution is given by Eq. (3). The comparison of a theoretical value with a measured one allows estimating the mass of water which transits in comparison to the mass of water present within the solution. Experimental values

lower than the theoretical ones materialize an evolution of the proportion of solution which is crystallized. Indeed, it materializes a deviation in the evolution of the compound concentrations within the remaining solution (according to the lever rule).

$$\Delta H_{\text{calculated with an inert mass}}([c]) = \Delta H_{\text{measured}}([c] = 0) * \Psi_{\text{IMV+5\%(v/v)Me}_2\text{SO}} \quad (3)$$

where $\Delta H_{\text{calculated with an inert mass}}([c])$ is the calculated specific enthalpy for the transition of liquid water to ice for an IMV + 5% (v/v) Me_2SO solution with a concentration [c] of inert mass ($J.g^{-1}$), $\Delta H_{\text{measured}}([c] = 0)$ is the experimentally measured specific enthalpy for the transition of liquid water to ice for a IMV + 5% (v/v) Me_2SO solution = 265.68 ± 3.2 $J.g^{-1}$ (this specific enthalpy value corresponds to the average of the three measurements with the IMV + 5% (v/v) Me_2SO solution without COS) ($J.g^{-1}$), and $\Psi_{\text{IMV+5\%(v/v) Me}_2\text{SO}}$ is the initial mass proportion of the IMV + 5% (v/v) Me_2SO solution in the prepared IMV + 5% (v/v) Me_2SO solution containing an inert mass (Ø).

Eq. (4) allows estimating the initial mass proportion of the IMV + 5% (v/v) Me_2SO solution in the prepared IMV + 5% (v/v) Me_2SO solution containing an inert mass such as COS.

$$\begin{aligned}\Psi_{\text{IMV+5\%(v/v)Me}_2\text{SO}}(\text{sol}) &= \frac{m_{\text{IMV+5\%Me}_2\text{SO}}}{m_{\text{IMV+5\%Me}_2\text{SO}} + m_{\text{product}}} \\ &= \frac{d_{\text{IMV+5\%Me}_2\text{SO}} * \rho_{H_2O} * V_{\text{sol}}}{d_{\text{IMV+5\%Me}_2\text{SO}} * \rho_{H_2O} * V_{\text{sol}} + [\text{product}] * V_{\text{sol}}} \\ &= \frac{1014.5}{1014.5 + [\text{product}]}\end{aligned} \quad (4)$$

where $\Psi_{\text{IMV+5\%(v/v) Me}_2\text{SO}}$ is the initial mass proportion of the IMV + 5% (v/v) Me_2SO solution in the prepared IMV + 5% (v/v) Me_2SO solution containing an inert mass (Ø), $d_{\text{IMV + 5\%Me2SO}}$ is the density of the IMV + 5%(v/v) Me_2SO solution = 1.0145 (experimentally measured with a pipette and a high sensibility weighing scale) (Ø), ρH_2O is the mass volume of water ≈ 1000 ($mg.mL^{-1}$), V_{sol} is the volume of the solution (mL), and [product] is the initial mass concentration of hydrated product added in solution ($mg.mL^{-1}$).

7.1.5 Estimation of the cryoscopic depression

A cryoscopic depression indicates the action of a dissolved product on the properties of water molecules in solution, providing information on interactions that occur between this product and the water molecules. The estimation of the maximal equilibrium temperature (T_m), for different concentrations of the same COS introduced in the IMV + 5% (v/v) Me_2SO solution, informs about the cryoscopic depression induced by the oligosaccharide. Moreover, knowing the temperature (T_m) of a solution allows to estimate, as a function of the temperature, the supercooling magnitude reached in this mixture (before crystallization).

Due to the kinetic phenomena of heat transfer, it is often imprecise to confuse the temperature recorded at the maximum of the endothermic melting peak (T_{max}) with the maximum melting point temperature of the ice in solution, because T_m is less or equal to T_{max} [119]. In the kinetic phenomena being influenced by the mass involved in the melting process, the comparison of the T_{max} values becomes difficult between the series for which the mass of sample studied is different and in which the mass of water in solution concerned by the melting is different.

Since the T_{max} values cannot be compared and can hardly be associated only with a cryoscopic phenomenon, we have not realized this comparison. We used an alternative method for estimating the cryoscopic effect based on the shape of the thermograms. It consists in the comparison of the size and the peaks spread with temperature [119].

7.2 Phase transition analysis of crystallizable water

The calculated values of the crystallization and melting enthalpies of the solutions were normalized to 0°C and represented in **Figure 1**. The calculation of the phase change enthalpy expected following the addition of an inert mass was performed in the range of the concentrations studied, using Eqs. (3) and (4). These values were plotted (**Figure 1**), with measured enthalpy values (normalized to 0°C), as a function of the initial COS concentration.

A sharp decrease in the ΔH_c and ΔH_m values is observed as a function of the mass concentration of COS introduced, and this decrease seems affine. For the same mass concentration of non-hydrated powder studied, the differences between ΔH_c and ΔH_m are small. Until 50 $mg.mL^{-1}$, a good correlation is observable between the calculated mass enthalpies following the addition of a non-hydrated inert mass and the measured mass enthalpies in the presence of COS (cf. **Figure 1**). COS molecules added in the solution can replace part of the water molecules present in the solution and thus reduce the amount of crystallizable water in mass proportion (as materialized by the orange line in **Figure 1**). To play an additional role, the CPA molecules must either bind to a portion of the water molecules to prevent their crystallization or promote the glass-forming tendency of free water during cooling. Based on **Figure 1**, COS do not seem to have significant effects, although these effects increase for the higher COS concentration (> 100 $mg.mL^{-1}$).

For the smallest COS concentrations, if the COS could bind to a part of the water, or highly favor the vitrification, then a difference between calculated enthalpies and experimental enthalpies or a difference between the crystallization enthalpies and the melting enthalpies (for a nominative mass of COS introduced in solution) would have been expected. These results imply that the addition of COS in

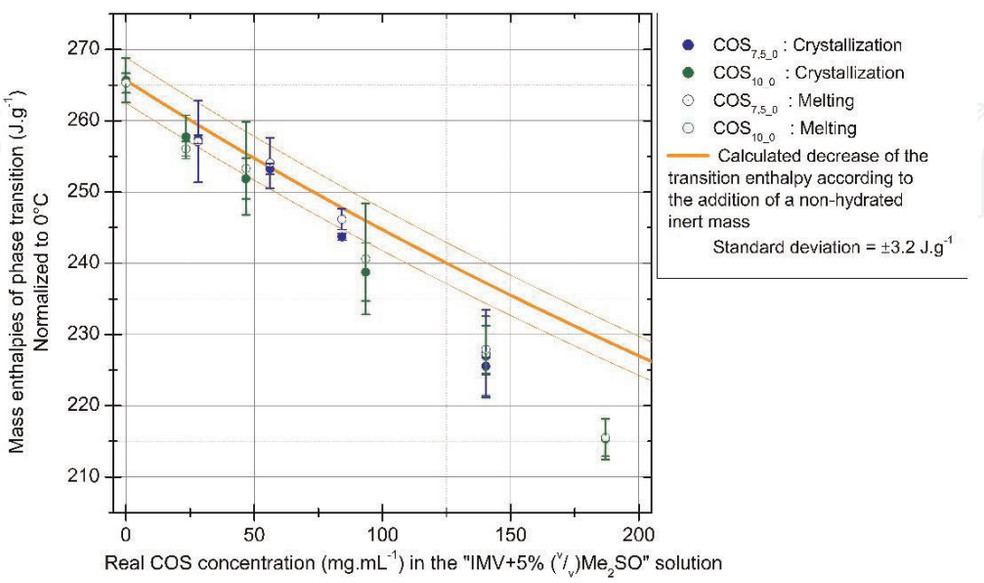

Figure 1.
Crystallization and melting enthalpies, normalized to 0°C, for the different solutions containing COS. Here the calculated transition enthalpies are also materialized in the case of the addition of a non-hydrated inert mass in solution. The standard deviations are calculated according to the three data obtained for each value. The standard deviation of the calculated enthalpy is the standard deviation of the measured value without COS.

the solution does not disturb further the ability of water molecules to crystallize. Thus, the COS presence does not alter either the amount of bounded water or the quantity of bulk water that vitrified.

For the highest COS concentrations, the amount of water that can crystallize is reduced, as indicated by the smaller normalized values of ΔH_c and ΔH_m. This decrease in crystallization and melting enthalpies implies a decrease in the amount of ice formed. Because normalized values are lower than the calculated, we argue that it is the materialization of the capability of COS to stabilize the remaining solution and to limit the crystallization of a part of the water molecules in the remaining solution.

COS, therefore, serve the purpose of reducing ice formation during cryopreservation procedures. However, their mode of action and its consequences for cells deserve to be studied deeper.

7.3 Cryoscopic depression induced by the addition of COS

As could be seen in **Figure 2**, when COS is added, the maximum of the heat flow related to the melting endothermic peaks appears to be smaller, and the peaks are shifted toward the low temperatures.

When there is little ice in the sample, the effects responsible for the temperature difference between T_m and T_{max} decrease. Thus, the decrease in the amount of ice formed when the amount of COS increases (cf. **Figure 1**) could explain the lowering of T_{max} (cf. **Figure 2**) without cryoscopic effect. When there is a smaller amount of ice and fewer phenomena that may imply the shift of the temperature of the

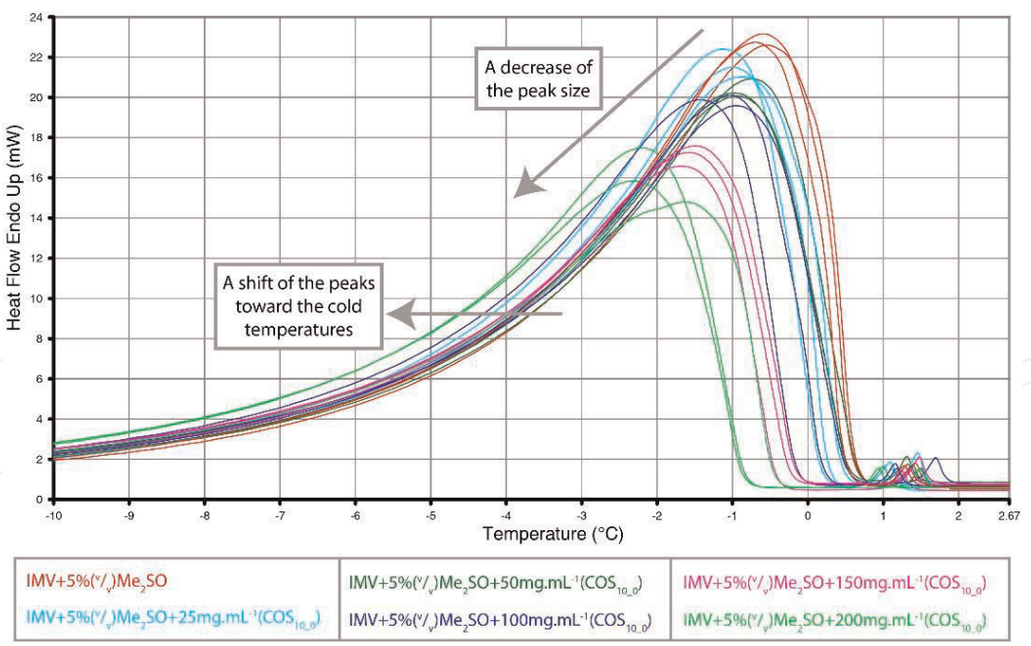

Figure 2.
Zoom on the bottom of the melting peaks obtained for the mixture: IMV + 5% (v/v) Me_2SO + [COS_{10_0}]. Observation of the COS influence on the shape of the melting peaks. Protocol, 2. Sample volume = 10 μL. Warming rate = +2.5°C.min^{-1}. Data range, normal. All cooling and warming thermograms were straightened up from −100 to −80°C and then lined up at 0.0 mW. The mass concentrations of the studied hydrated powder are equal to 0, 12.5, 25, 100, and 200 mg.mL^{-1}. Two endothermic peaks are observable on each of these thermograms, a large one which is associated to the melting of the ice previously crystallized within the sample and a small one which is an anomaly. This anomaly of melting is systematically present during the analysis of slow-freezing solutions, linked to the melting of the previously condensed and crystallized ambient humidity of the air encapsulated within the sample pan. This humidity could be from the previous ambient air in the laboratory or from the equilibration at the vapor pressure of water between the encapsulated air and the sample aqueous solution or from a combination of both.

melting peak summit, the melting peak is smaller, and then, the temperature of the peak's summit is shifted toward low temperatures.

However, the offset that is observed at the onset of the peaks cannot be associated with a reduction in the amount of ice that melts during warming. This shift indicates that there is a change in the amount of ice transiting at lower temperatures. The amount of water that melts as a function of temperature is greater at lower temperatures for the solutions with COS (cf. **Figure 2**), even if, in the presence of COS, the total amount of ice previously formed is lower. This phenomenon is the materialization of a slight cryoscopic effect linked to the addition of COS in solution. This effect is, however, difficult to quantify and remains below 0.5°C (cf. **Figure 2**).

The presence of a weak cryoscopic depression associated with the addition of COS in the IMV + 5% (v/v) Me_2SO solution involves the addition of COS having little or no influence on the osmolality of the studied solutions. Further verifications will nevertheless have to be made to verify these results, especially by studying the characteristics of the mixture: "H_2O-COS." Based on these preliminary findings, we will consider that COS compounds can be added to cryopreservation solutions without fear of a significant increase or acceleration of cell dehydration.

8. Conclusion on the COS usage for cryopreservation

According to this first thermodynamic characterization, the use of COS as CPA is debatable, as are their cryoprotective capabilities.

COS appear to be biocompatible in solution, and no disruption to mouse embryo development has occurred when COS were evaluated. COS have a significant influence on lowering the amount of crystallizable ice in solution, without participating in cell dehydration. Indeed, their addition in solution replaces a significant part of water, without significant osmotic effect. This is an interesting use in slow-freezing cryopreservation since it makes it possible to reduce the mechanical damage associated with the formation of ice, without change in the equilibrium of the osmotic balance with the intracellular media. We can thus assume that the use of such a product does not promote the mechanisms of dehydration of the intracellular medium.

However, COS do not seem able to influence the proportion of IMV + 5% (v/v) Me_2SO solution remaining liquid during freezing. As a result, they cannot participate in the main protections associated with CPA. Using these compounds without any other CPA has, therefore, to be discarded. COS do not participate in the equilibration of osmotic pressures between intra- and extracellular media. Thus, they dehydrate the overconcentrated residual solution, without modifying the mass proportion of crystallized water and without dehydrating the cells. They act only in the extracellular medium by inducing a steric hindrance and by increasing the solution viscosity. They seem to contribute to the stabilization of the extracellular noncrystalline medium.

Combined with penetrating cryoprotectants, we assume that COS can promote a decrease in the use of penetrating CPA while ensuring the successful vitrification of intercrystalline spans. Nevertheless, several questions remain about the action of COS in solution. It is particularly possible to question their interactions with water molecules. Similarly, it is difficult to know how these compounds interact with Me_2SO. In order to further characterize the role of these compounds in water solution, it will be necessary to characterize them in simpler binary solutions composed of COS and water. It would also be necessary to study the influence of COS on the ice crystal organization in cryopreserved samples (notably to study the

influence of COS on the average size of intercrystalline spans). Further experiments, particularly on their ability to increase the glass-forming tendency of the remaining solution, should be conducted in the future.

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