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MINI-REVIEW

Dimethyl sulfoxide in cryopreservation: An overview

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Abstract: Cryopreservation is a process that preserves intact living cells, tissues, or any other biological constructs by cooling and storing the samples to very low temperatures in order to maintain their viability and to save them for future use. At very low temperatures, enzymatic and chemical activities that may cause harm to the biological material are effectively stopped. Cryopreservation methods look to reach very low temperatures without causing further harm caused by the creation of ice crystals during freezing. Cryopreservation of human samples for storage including a variety of diseases such as cancer, genetic and degenerative disorders and bio-banking. This review aims to describe and highlight dimethyl sulfoxide in cryopreservation. Dimethyl sulfoxide is a non-toxic solvent and is one of the most commonly used pharmaceutical agents with a varied range of pharmacological actions including membrane penetration, anti-inflammatory effects and local analgesia as well as weak bacteriostatic. The main use of dimethyl sulfoxide is a vehicle for some drugs to enhance the action of the drug and help penetration of other drugs into the skin. However, dimethyl sulfoxide has been the cryopreservative agent of choice in situations due to its exceptional performance in mitigating freezing-related damages. It is frequently used in cell banking applications as a cryoprotectant. When added to media, it prevents intracellular and extracellular crystals from forming in cells during the freezing process. There has been a concern over the toxicity of dimethyl sulfoxide and its potential side effects in different high concentrations after administration to patients. Therefore, there has been a growing demand for robust dimethyl sulfoxide for cryopreservation methods that can improve safety and maintain potency and efficacy.

Introduction

Dimethyl sulfoxide (DMSO) is an organosulfur compound with the formula of $(CH_3)_2SO$. This colorless liquid is an important polar-aprotic solvent that dissolves polar and non-polar compounds and it is miscible in a wide range of organic solvents as well as in water [1]. It also has a relatively high melting point. DMSO has the unusual property that many individuals perceive a garlic-like taste in the mouth after contact with the skin. In terms of chemical structure, the molecule has idealized Cs symmetry. It has trigonal pyramidal molecular geometry consistent with the other three-coordinate S(IV) compounds (**Figures 1 and 2**), with a non-bonded

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electron pair on an approximately tetrahedral sulfur atom [2]. Because of its ability to dissolve many kinds of compounds, DMSO plays a role in sample management and high-throughput screening operations in drug design [3, 4]. In biology, DMSO is used in a polymerase chain reaction (PCR) to inhibit secondary structures in the DNA template or the DNA primes (stem-loop intramolecular base pairing is a pattern that can occur in single-stranded DNA or, more commonly, in RNA. The structure is also known as a hairpin or loop. It occurs when two regions of the same strand, usually complementary in nucleotide sequence when read in opposite directions, base-pair to form a double helix that ends in an unpaired loop [5]. The resulting structure is a key building block of many RNA secondary structures. It is added to the PCR mix before reacting, where it interferes with the self-complementarity of the DNA, minimizing interfering reactions. DMSO in PCR reaction is applicable for supercoiled plasmids (to relax before amplification) or DNA templates with high GC-content- (or guanine-cytosine content) - (to decrease thermostability) [6]. For example, 10.0% of the final concentration of DMSO in the PCR mixture with a Phusion decreases primer annealing temperature (i.e. primer melting temperature) by 5.5-6.0°C [7].

It is well known as a reversible cell cycle arrester at phase G1 of human lymphoid cells [6]. DMSO may also be used as a cryoprotectant, added to cell media to reduce ice formation and thereby prevent cell death during the freezing process [8]. About 10.0% (as a higher concentration) may be used with a slow-freeze method and the cells may be frozen at -80 °C or stored in liquid nitrogen safely. DMSO has been used as a co-solvent to assist absorption of the flavonol glycoside Icariin into the C.elegans nematode worm [9]. In cell culture, DMSO is used to induce differentiation of P19 embryonic carcinoma cells into cardiomyocytes and skeletal muscle cells [10]. Frozen is a non-toxic solvent and is one of the most commonly used pharmaceutical agents with a varied range of pharmacological actions such as membrane penetration, anti-inflammatory effects, local analgesia and weak bacteriostatic [11]. The main use of frozen is a vehicle for some drugs to enhance the action of the drug and help penetration of other drugs into the skin. However, frozen has been the cryopreservative agent of choice in a situation due to its exceptional performance in mitigating freezing-related damages. It is frequently used in cell banking applications as a cryoprotectant.

Reactions with electrophiles: The sulfur center in DMSO is nucleophilic toward soft electrophiles and the oxygen is nucleophilic toward hard electrophiles. In addition, it forms with methyl iodide a compound of trimethylsulfoxonium iodide, $[(CH_3)_3SO]$ [1, 2].

$$(CH_3)_2SO + CH_3I \rightarrow [(CH_3)_3SO].I$$

This salt can be deprotonated with sodium hydride to form the sulfurylide:

$$[(CH_3)_3SO]I + NaH \rightarrow (CH_3)_2S(CH_2)O + NaI + H_2$$

Acidity: The methyl groups of DMSO are only weakly acidic with a pKa=35. For this reason, the basicities of many weakly basic organic compounds have been examined in this solvent deprotonation of DMSO which requires strong bases like lithium di-isopropylamide and sodium hydride. Stabilization of the resultant carbanion is provided by the S(O)R group. The sodium derivative of DMSO formed in this way is referred to as dimsylsodium. It is a base, e.g., for the deprotonation of ketones to form sodium enolates, phosphonium salts to form Wittig reagents and formamidinium salts to form diaminocarbenes. It is also a potent nucleophile. DMSO is a polar aprotic solvent and is less toxic than other members of this class such as dimethylformamide, dimethyl-acetamide, N-methyl-2-pyrrolidone and HMPA. DMSO is frequently used as a solvent for chemical reactions involving salts, most notably Finkelstein reactions and other nucleophilic

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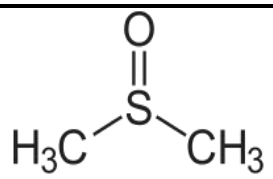
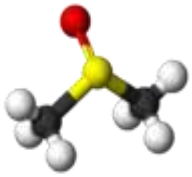
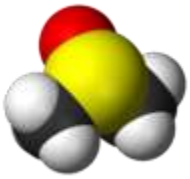
substitutions. In addition, it is extensively used as an extractant in biochemistry and cell biology [13]. Because DMSO is only weakly acidic, it tolerates relatively strong bases and has extensively been used in the study of carbanions. A set of non-aqueous pKa values (C-H, O-H, S-H and N-H acidities) for thousands of organic compounds have been determined in DMSO solution [1, 2]. Because of its high boiling point, 189°C, DMSO evaporates slowly at normal atmospheric pressure.

Dimethyl sulfoxide (**DMSO**)	
	
IUPAC name Dimethyl sulfoxide	 A sample of dimethyl sulfoxide
	Systematic IUPAC name Methanesulfinylmethane (substitutive) Dimethyl(oxide) sulfur (additive) Other names are Methylsulfinylmethane, Methyl sulfoxide

Properties	
Chemical formula	C_2H_6OS
Molar mass	78.13 g per mol
Appearance	Colorless liquid
Density	1.1004 g cm^{-3}
Melting point	19 °C (66 °F; 292 K)
Boiling point	189 °C (372 °F; 462 K)
Solubility in water	Miscible
Solubility in diethyl ether	Very soluble
Acidity (pK_a)	35
Refractive index (n_D)	1.479 ε_r = 48
Viscosity	1.996 cP at 20 °C

Figure 1: Chemical and physical properties of DMSO

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Samples dissolved in DMSO cannot be as easily recovered compared to other solvents, as it is very difficult to remove all traces of DMSO by conventional rotary evaporation. One technique to fully recover samples is removal of the organic solvent by evaporation followed by the addition of water (to dissolve DMSO) and lyophilisation to the remove DMSO and water. Reactions conducted in DMSO are often diluted with water to precipitate or phase-separate products. The relatively high freezing point of DMSO, 18.5 °C, means that at, or just below, room temperature it is a solid, which can limit its utility in some chemical processes such as crystallization with cooling.

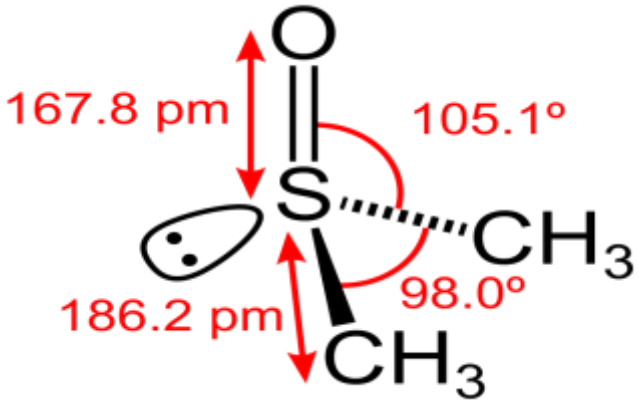

Figure 2: Geometric molecular measurements of DMSO [12]

In its deuterated form (DMSO-d6), it is a useful solvent for NMR spectroscopy, again due to its ability to dissolve a wide range of analytes, the simplicity of its own spectrum and its suitability for high-temperature NMR spectroscopic studies. Disadvantages to the use of DMSO-d6 are its high viscosity, which broadens signals and its hygroscopicity, which leads to an overwhelming H_2O resonance in the 1H NMR spectrum. It is often mixed with $CDCl_3$ or CD_2C_2 for lower viscosity and melting points. DMSO is finding increased use in manufacturing processes to produce microelectronic devices [14]. It is widely used to strip photoresist in TFT-LCD 'flat panel' displays and advanced packaging applications (wafer-level packaging/ solder bump patterning). It is also used in bio-preservation especially stem cell banking. DMSO is an effective paint stripper, being safer than many of the others such as nitromethane and dichloro-methane. Because of its ability to dissolve many kinds of compounds, DMSO plays a role in sample management and high-throughput screening operations in drug design [1].

Biological activity: DMSO is used in PCR to inhibit secondary structures in the DNA template or the DNA primers. It is added to the PCR mix before reacting, where it interferes with the self-complementary of the DNA, minimizing interfering reactions [15]. DMSO in a PCR reaction is applicable for supercoiled plasmids (to relax before amplification) or DNA templates with high GC-content (to decrease thermostability). For example, a 10.0% final concentration of DMSO in the PCR mixture with Phusion decreases primer annealing temperature (primer melting temperature) by 5.5-6.0°C [5, 15]. It is well known as a reversible cell cycle arrester at phase G1 of human lymphoid cells [16]. DMSO may also be used as a cryoprotectant, added to cell media to reduce ice formation and thereby prevent cell death during the freezing process [14, 15, 17]. About 10.0% may be used with a slow-freeze method, and the cells may be frozen at -80°C or stored in liquid nitrogen safely [18]. DMSO has been used as a co-solvent to assist absorption of the flavanol glycoside Icariin into the C. elegans nematode worm [11]. In cell culture, DMSO is used to induce differentiation of P19 embryonic carcinoma cells into

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cardiomyocytes and skeletal muscle cells. In cryobiology, DMSO has been used as a cryoprotectant and is still an important constituent of cryoprotectant vitrification mixtures used to preserve organs, tissues and cell suspensions. Without it, up to 90.0% of frozen cells will become inactive. It is particularly important in the freezing and long-term storage of embryonic stem cells and hematopoietic stem cells which are often frozen in a mixture of 10% DMSO, a freezing medium and 30.0% fetal bovine serum. In the cryogenic freezing of heteroploidy cell lines (MDCK, VERO, etc.) a mixture of 10.0% DMSO with 90.0% EMEM (70.0% EMEM plus 30.0% fetal bovine serum plus antibiotic mixture) is used. As part of an autologous bone marrow transplant, the DMSO is re-infused along with the patient's own hematopoietic stem cells [3, 19, 20].

Cryopreservation: The best method for cryopreserving cultured cells is storing them in liquid nitrogen in a complete medium in the presence of cryoprotective agent such as DMSO. Cryoprotective agents reduce the freezing point of the medium and also allow a slower cooling rate, greatly reducing the risk of ice crystal formation, which can damage cells and cause cell death. DMSO is known to facilitate the entry of organic molecules into tissues. Handle reagents containing DMSO using equipment and practices appropriate for the hazards posed by such materials. Dispose of the reagents in compliance with local regulations.

Guidelines for cryopreservation: Following the guidelines below is essential for cryopreserving the cell lines for future use. As with other cell culture procedures, it is recommended that a closely follow the instructions provided with the cell line for best results: Freeze the cultured cells at a high concentration and at as low a passage number as possible. Make sure that the cells are at least 90.0% viable before freezing. Note that the optimal freezing conditions depend on the cell line in use. Freeze the cells slowly by reducing the temperature to about 1°C per minute using a controlled rate cryo-freezer or a cryo-freezing container such as Mr. Frosty, available from NALGENER® labware (Nagle Nunc) [15]. Use the recommended freezing medium. The freezing medium should contain cryoprotective agents such as DMSO or glycerol. Store the frozen cells below -70°C; frozen cells begin to deteriorate above -50°C. Use the sterile cryovials for storing frozen cells. Cryovials containing the frozen cells may be stored immersed in liquid nitrogen or in the gas phase above the liquid nitrogen. Wear personal protective equipment. All the solutions and equipment that come in contact with the cells must be sterile. Finally, use a proper sterile technique and work in a laminar flow hood [8].

Freezing medium: Use the recommended freezing medium for cryopreserving your cells. The freezing medium should contain a cryoprotective agent such as DMSO or glycerol. It can also use a specially formulated complete cryopreservation medium such as Recovery™ cell culture freezing medium or Synth-a-Freezer cryopreservation medium. Reco-very™ cell culture freezing medium is a ready-to-use complete cryopreservation medium for mammalian cell cultures, containing an optimized ratio of fetal bovine serum to bovine serum for improved cell viability and cell recovery after thawing. Synth-a-FreezeR cryopreservation medium is a chemically defined, protein-free, sterile cryopreservation medium containing 10.0% DMSO that is suitable for the cryopreservation of many stem and primary cell types with the exception of melanocytes [3, 8, 14, 15, 17].

Materials: Culture vessels containing cultured cells in the log-phase of growth. Complete growth medium. Cryoprotective agents such as DMSO (use bottle set aside for cell culture; open only in a laminar flow hood) or a freezing medium such as Synth-a-FreezeR cryopreservation medium or Recovery™ cell culture freezing medium. Disposable, sterile 15 mL or 50 mL conical tubes. Reagents and equipment to determine viable and total cell counts (Countess R). Automated cell counter, or the hemacytometer, cell counter and Trypan Blue). Sterile cryogenic storage vials (cryovials). Controlled rate freezing apparatus or isopropanol chamber. Liquid nitrogen

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storage container for freezing adherent cells, in addition to the above materials, balanced salt solution such as Dulbecco's phosphate buffered saline (D-PBS), containing no calcium, magnesium or phenol red [8, 13, 14, 17].

Protocol for cryopreserving cultured cells: The following protocol describes the general procedure for cryopreserving cultured cells. For detailed protocols, always refer to the cell-specific product insert. Prepare freezing medium and store between 2°C and 8°C until use. The appropriate freezing medium depends on the cell line [17]. For adherent cells, gently detach cells from the tissue culture vessel following the procedure used during the subculture. Re-suspend the cells in the complete medium required for that cell type. Determine the total number of cells and percent viability using a hemacytometer, cell counter and Trypan Blue exclusion, or the Countess® automated cell counter. According to the desired viable cell density, calculate the required volume of the freezing medium. Centrifuge the cell suspension at about 100-200 g for 5.0-10.0 minutes aseptically decant supernatant without disturbing the cell pellet (centrifugation speed and duration vary depending on the cell type). Re-suspend the cell pellet in the cold freezing medium at the recommended viable cell density for the specific cell type. Dispense aliquots of the cell suspension into cryogenic storage vials. As you aliquot them, frequently and gently mix the cells to maintain a homogeneous cell suspension. Freeze the cells in a controlled rate freezing apparatus, decreasing the temperature about 1°C per minute. Alternatively, place the cryovials containing the cells in an isopropanol chamber and store them at -80 °C overnight. Transfer frozen cells to liquid nitrogen and store them in the gas phase above the liquid nitrogen. Dissociation reagents such as trypsin or TrypLE™ express without phenol red [14, 15].

Guidelines for thawing: The thawing procedure is stressful to frozen cells and using good technique and working quickly ensures that a high proportion of the cells survive the procedure. As with other cell culture procedures, it is recommended to closely follow the instructions provided with the cells and other reagents for the best results. Thaw frozen cells rapidly (<1 min) in a 37°C water bath. Dilute the thawed cells slowly, using pre-warmed growth medium. Plate thawed cells at a high density to optimize recovery. Always use proper aseptic technique and work in a laminar flow hood. Always wear personal protective equipment, including a face mask or goggles. Cryovials stored in the liquid-phase present a risk of explosion when thawed. Some freezing media contain DMSO which is known to facilitate the entry of organic molecules into tissues. Handle reagents containing DMSO using equipment and practices appropriate for the hazards posed by such materials.

Conclusion: DMSO in a low concentration may increase the rate of apoptosis in the cell cycle G1 phase. However, this assumption should be tested in-vivo conditions before the final assumption. DMSO is usually used as a cryoprotective agent during the freezing of cells for storage at 10.0%, although more recent data suggests a concentration of about 05.0% may be more effective. Different concentrations of DMSO are suitable for cryopreservation but further studies are required to establish a proper concentration to use in each type of culture media.

Conflict of interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
Ethical issues: Including plagiarism, informed consent, data fabrication or falsification, and double publication or submission were completely observed by the author.
Data availability statements: The raw data that support the findings of this article are available from the corresponding author upon reasonable request.

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