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Development and Optimization of Novel Single Mode Electromagnetic Resonant (SMER) Techniques for Cryopreservation

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Abstract

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(SMER) Techniques for Cryopreservation

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Since 1949, scientists have made significant efforts to advance cryopreservation techniques for a wide range of biological materials, including deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins, biofluids, cells, tissues, and organs. Cryopreservation typically involves five major steps: cryoprotective agent (CPA) loading, cooling, storage, warming, and CPA unloading. Despite decades of progress, the field still relies heavily on manual, dropwise CPA addition and removal methods. Current dilution protocols often employ isotonic solutions or multi-step dilution approaches, which are labor-intensive and may not be optimized for different cell types. There is a critical need for fully automated systems that can perform CPA addition and removal in a

controlled, cell-type-specific manner—taking into account factors such as volumetric flow rate and hypotonic solution selection to minimize osmotic stress. Furthermore, a post-dilution concentration step that eliminates the need for centrifugation would greatly enhance the clinical utility of cryopreserved cell suspensions, particularly in cellular therapy applications.

For the cooling process, vitrification offers an effective strategy to prevent ice crystal formation, which is particularly important for preserving complex biological systems such as tissues and organs containing multiple cell types. However, conventional vitrification typically requires high concentrations of small-molecule cryoprotectants like dimethyl sulfoxide (DMSO) or glycerol, which can cause significant osmotic and toxic injury to cells and tissues. To address this challenge, recent efforts have focused on reducing the use of small-molecule CPAs in vitrification protocols. In this work, we introduce a novel approach utilizing high concentrations of polyvinylpyrrolidone (PVP) with varying molecular weights to minimize DMSO content while maintaining vitrification efficacy. This strategy demonstrated successful preservation outcomes in both cell suspensions and tissue samples. Preliminary experiments on mouse liver tissue confirmed the feasibility of this CPA formulation. Using this protocol, 4 mL of cell suspension could be directly plunged into liquid nitrogen for vitrification and subsequently rewarmed in a water bath, resulting in high post-thaw recovery and proliferation rates.

Conventional water bath rewarming is inadequate for large vitrified samples due to its reliance on low-efficiency convective heat transfer. In contrast, electromagnetic (EM) waves can penetrate biological materials and enable volumetric heating, offering a more effective solution for rapid and uniform rewarming. To optimize EM-based rewarming, three key parameters must be considered: operating frequency, dielectric properties of the sample, and electric field intensity (determined by input power). A single-mode electromagnetic resonant (SMER) cavity was

developed to concentrate energy through mode selection, enabling efficient and localized energy deposition. A generalized design methodology was established for constructing both electric and magnetic field–dominant cavities at their respective fundamental modes. To address the frequency drift caused by temperature-dependent changes in dielectric properties, an auto-resonance tracking system was implemented to maintain resonance during the warming process. This system also monitors the voltage standing wave ratio (VSWR) to quantify reflected power. The complete system was successfully applied to rewarm both 10 mL and 20 mL frozen and vitrified samples, demonstrating superior performance compared to conventional rewarming techniques.

In addition to cell suspensions, this work extends to the cryopreservation of tissues and organs. Plans for future development are also outlined in this dissertation, including the advancement of next-generation cryoprotective agents, the design of an integrated perfusion system, and the construction of an improved electromagnetic cavity. The proposed cavity will incorporate features such as real-time temperature monitoring, user-friendly sample loading, antenna coupling optimization, electric field uniformity enhancement, and an integrated shaking platform to promote thermal homogeneity. Ultimately, this research aims to advance toward the successful cryopreservation and rewarming of whole organs.

TABLE OF CONTENTS

Chapter 1. Introduction	1
1.1 Cryobiology and cryopreservation.....	1
1.1.1 Slow freezing cooling	2
1.1.2 Vitrification cooling	3
1.2 Current available method in rewarming	6
1.2.1 Conventional methods	6
1.2.2 High intensity focus ultrasound	10
1.2.3 Electromagnetic rewarming theory	12
1.3 Challenges in rewarming	24
1.4 Dissertation outline	26
Chapter 2. DEVELOPMENT OF A 3-IN-1 MULTIFUNCTIONAL CELL PROCESSING SYSTEM AND OPTIMIZATION OF CELL TYPE-DEPENDENT PROTOCOLS FOR CPA ADDITION/REMOVAL	29
2.1 Introduction.....	29
2.2 Material and methods.....	32
2.2.1 Design and setup	32
2.2.2 Numerical simulation in selection of flow rate, dilution solution, and processing time	34
2.2.3 Cell-free CPA removal experiment	37
2.2.4 Cell concentrating	38
2.3 Results.....	39
2.3.1 Numerical results and experiment results for CPA removal	39
2.3.2 Numerical results and experiment results for cell concentrating	45
2.4 Discussion	45
2.5 Conclusion	48
Chapter 3. DEVELOPMENT OF NEW VITRIFICATION SOLUTION BY INTRODUCING POLYVINYLPYRROLIDONE	49

3.1 Introduction.....	49
3.2 Material preparation.....	50
3.3 Determination of the thermal properties of CPA solutions	51
3.3.1 Heat Capacity	51
3.3.2 Glass transition temperature	52
3.3.3 Results and Discussion	53
3.4 Determination of Viscosity of CPA solutions	55
3.4.1 Viscosity measurement	55
3.4.2 Results and Discussion	56
3.5 Determination of osmolality of CPA solutions.....	57
3.5.1 Osmolality calculation	57
3.5.2 Osmolality measurement	58
3.5.3 Results and discussion	59
3.6 Determination of Dielectric Properties of CPA solutions	59
3.6.1 Coaxial probe measurement for CPAs in broadband frequency (200 MHz – 1.5 GHz) at room temperature	60
3.6.2 Cavity perturbation method for CPAs at subzero temperature	61
3.6.3 Results and discussion	63
3.7 Determination of biocompatibility.....	66
3.7.1 Toxicity Injury Measurement of PVP and DMSO	66
3.7.2 Results and discussion	67
3.8 Summary	69
Chapter 4. IMPROVED CRYOPRESERVATION OF JURKAT CELL SUSPENSIONS USING A NOVEL CPA IN FREEZING AND VITRIFICATION CONDITIONS	70
4.1 Introduction.....	70
4.2 Material and methods.....	71
4.2.1 Sample preparation	71
4.2.2 Cooling device and process	72
4.2.3 Rewarming methods	72
4.2.4 Temperature monitoring	72
4.2.5 Recover rate of the Jurkat cells	73

4.2.6 Assessment of the post-thaw biological metabasin	73
4.3 Results and discussion	74
4.3.1 Determination of the recovery rate of the Jurkat cells	74
4.3.2 Determination of the metabolic activity of the post-thawed cells	75
4.4 Conclusion	77
Chapter 5. DEVELOPMENT AND OPTIMIZATION OF A BROADBAND SINGLE-MODE ELECTROMAGNETIC RESONANCE CAVITY INTEGRATED WITH AUTO-RESONANT TRACKING	79
5.1 Introduction	79
5.2 Theory of single mode resonant cavity design	80
5.2.1 Development of the TE101 resonant cavity	82
5.2.2 Development of the TE102 resonant cavity	84
5.2.3 Critical rewarming rate guided maximum sample volume in a given cavity	85
5.2.4 Development of TE101 and TE102 resonant cavity for 900-930 MHz solid state generator	87
5.2.5 Development of the TE101 reflected cavity	88
5.3 Prototype design and optimization	89
5.3.1 Improvement of 434 MHz resonant cavity system design	89
5.3.2 System design of the 915 MHz TE101 resonant and reflected cavity	90
5.3.3 Design of auto-resonant tracking system	92
5.4 Simulation	93
5.5 Experiment	95
5.5.1 Validation of the auto-resonant tracking performance	95
5.5.2 Validation of SMER heating in vitrified sample	96
5.6 Result of cavity design	97
5.6.1 Development of TE101 cavity: 915MHz study example	97
5.6.2 Development of TE102 cavity: 915MHz study case	98
5.6.2 Development of TE101 reflected cavity: 915MHz study case	99
5.6.3 Unload cavity resonant frequency guided by material dielectric property for 910 – 930 MHz SSG	100

5.6.4 Determination of maximum sample volume: 434 MHz TE101 resonant cavity with DPVP	101
5.7 Result of cavity simulation	102
5.7.1 TE101 resonant cavity	102
5.7.2 TE101 Reflected Cavity.....	103
5.7.3 TE102 rewarming	103
5.8 Results of Experimental Rewarming	104
5.8.1 Comparison of tracking and untracking resonant frequency rewarming.....	104
5.8.2 Comparison of frozen and Vitrified rewarming.....	105
5.9 Discussion.....	106
5.10 Conclusion	108
Chapter 6. STUDY OF REWARMING DYNAMICS IN 4 ML AND 20 ML VITRIFICATION SOLUTIONS FOR VARIOUS POWER INPUT	109
6.1 Introduction.....	109
6.2 Material and methods.....	109
6.3 Results and Discussion	110
6.3.1 20mL solution experiment at 434 MHz	110
6.3.2 4mL solution experiment at 915 MHz	115
6.4 Conclusion	117
Chapter 7. ELECTROMAGNETIC REWARMING OF LARGE FROZEN AND VITRIFIED JURKAT CELL SUSPENSION.....	118
7.1 Introduction.....	118
7.2 Material and methods.....	118
7.2.1 10 mL cell suspension experiment.....	118
7.2.2 20 mL cell suspension experiment.....	118
7.2.3 Rewarming methods	119
7.2.4 Assessment methods	119
7.3 Results and discussion	120
7.3.1 Determination of the recovery rate of the Jurkat cells	120
7.3.2 Determination of the metabolic activity of the post-thawed cells	121

7.4 Conclusion	122
Chapter 8. Summary of future work	123
8.1 Summary	123
8.2 Future work	125
Appendix I	129
Reference	131

LIST OF FIGURES

Figure 1-1. Cryopreservation standard procedures.....	2
Figure 1-2. Peter Mazur’s “Two-factor” hypothesis.....	3
Figure 1-3. Frozen kidney vs. vitrified kidney by Gregory Fahy [27]	4
Figure 1-4. Energy conversion sources and methods in cryopreservation.	6
Figure 1-5. Illustration of the conventional heating method in cryopreservation.....	8
Figure 1-6. Heating mechanism of laser rewarming and its setup.....	13
Figure 1-7. Dielectric property resonant status of material versus frequency relationship.	13
Figure 1-8. Schematic diagram of the joule heating setup.....	15
Figure 1-9. Molecular structure of water (left), DMSO (middle), and glycerol (right)....	15
Figure 1-10. Configuration of capacitor heating.....	18
Figure 1-11. Schematic drawing of the antenna heating.....	19
Figure 1-12. Eddy Current illustration.....	21
Figure 1-13. Magnetic Hysteresis Effect and Nanorewarming Results.....	23
Figure 2-1. Diagram of the multifunctional cell processing system with three function flow diagrams.....	33
Figure 2-2. Simulation of CPA removal process.....	40
Figure 2-3. Cell volume change with different CPA removal methods.	42
Figure 2-4. Volume flow rate effect on CPA removal.....	43
Figure 2-5. Various CPA removal simulation with initial hypertonic solution.....	44
Figure 2-6. Cell concentrating mode of the multifunctional cell processing system between experiment and theoretical results using Jurkat cell as representation.	45
Figure 3-1. DSC equipment and its specification.....	52
Figure 3-2. Heat capacity of 10D50PVP (10kDa).....	53
Figure 3-3. Heat flow diagram and highlighted T_g of 10D50PVP	54
Figure 3-4. Discovery HR30 rheometer during operation.....	55
Figure 3-5. Rheometer procedure and setting parameter.....	56
Figure 3-6. Vtrification solution viscosity with different PVP MW and concentration...	57
Figure 3-7. Setup of Osmometer.....	58
Figure 3-8. Measured Solution heatmap using coaxial cable method	60
Figure 3-9. Setup of coaxial cable measurement of dielectric properties.....	61

Figure 3-10. Dielectric properties of pure and binary CPA solutions at room temperature	64
Figure 3-11. Determination of shape factor and calibration of the cavity perturbation method	64
Figure 3-12. Dielectric constant (top) and loss (bottom) of vitrification solutions	65
Figure 3-13. Dielectric constant (top) and loss (bottom) of vitrification solutions	66
Figure 3-14. alamar Blue Test of PVP group (left) and DPVP group (right). The concentration of each row is listed in Table 3-6	67
Figure 3-15. Absorbance difference in various time for 10 kDa PVP (up) and DPVP (bottom) group	68
Figure 4-1. Determination of the metabolic activity of the post-thawed Jurkat cells	74
Figure 4-2. The recovery rate of the post-thawed Jurkat cells	74
Figure 4-3. Incubating the cryopreserved and rewarmed cell	75
Figure 4-4. Determination of the post-thawed cells metabolic activity by alamarBlue	76
Figure 5-1. Geometry of the parallel waveguide	81
Figure 5-2. Electric and magnetic field of TE ₁₀₁ mode.	82
Figure 5-3. The schematic diagram of the updated SMER heating system.	90
Figure 5-4. System design of the resonant (left) and reflected (right) cavity	91
Figure 5-5. 915 MHz cavity with SSG	91
Figure 5-6. Design of RF auto-tracking system	92
Figure 5-7. 434MHz SMER system with auto-tracking system included.	93
Figure 5-8. Schematic of the simulated electromagnetic resonant rewarming system	94
Figure 5-9. Meshed computation model of the resonant cavity	95
Figure 5-10. TE ₁₀₁ cavity design parameter	97
Figure 5-11. Electric field (left) and magnetic field (right) distribution at 915MHz TE ₁₀₁ resonant cavity	98
Figure 5-12. TE ₁₀₂ cavity dimension design.	98
Figure 5-13. Top view magnetic field distribution of the TE ₁₀ cavity at different a, b, and d values.	99
Figure 5-14. Top view of electric field distribution of the reflected cavity at different d values.	100
Figure 5-15. 3-D electric field distribution of the reflected cavity at designed d = 22m	100

Figure 5-16. Unload cavity resonant frequency versus the sample volume and the dielectric constant	101
Figure 5-17. Maximum sample volume versus the critical warming rate (CWR).....	102
Figure 5-18. Simulated Results of 915MHz resonant cavity.	103
Figure 5-19. Simulated Results of 915MHz reflected cavity.	103
Figure 5-20. Simulation of TE102 magnetic heating.....	104
Figure 5-21. Temperature profile of tracking (n = 3) and untracking SMER system at 434MHz	105
Figure 5-22. Vitrified Sample (left) and frozen Sample (right).....	105
Figure 5-23. Temperature profile of vitrified and crystal sample starting from -75°C ...	106
Figure 6-1. 4 mL and 20 mL sample holder and optic fiber temperature sensor setup ..	110
Figure 6-2 Vitrified sample after cooled to 150°C at virous cooling rates.	111
Figure 6-3 Vitrification solution directly Plugged into LN2 tank showing crack.	111
Figure 6-4 Temperature profile for water bath and auto tracking SMER.....	111
Figure 6-5 Temperature profile of edge (left) and center (right) for various vitrified sample rewarmed by auto-tracking SMER.	112
Figure 6-6 Various power input using auto-tracking SMER for 20D30PVP (58kDa)...	113
Figure 6-7 Resonant frequency change (up-left), (up-right) voltage standing wave ratio (VSWR) , and reflects power (bottom-left) for vitrification samples during the rewarming process.	114
Figure 6-8. 4 mL vitrified sample rewarmed by 915 MHz SMER system with auto-tracking at 350 W (left) and 1 kW (right)	115
Figure 6-9 Various power for 4mL 10D37.5PVP (10 kDa) rewarming	115
Figure 6-10. Resonant frequency and VSWR change over temperature using 10D37.5PVP (10kDa) as example.	116
Figure 6-11. Resonant frequency and reflected power change of 915 MHz during rewarming	116
Figure 8-1. Mice liver vitrification.	126

LIST OF TABLES

Table 1-1. Water Bath Rewarming Experiments of Cryopreserved Samples	9
Table 2-1. Control settings for different working modes	34
Table 2-2. Setup of Cell-free CPA Removal Experiments	38
Table 2-3. Cell properties and membrane permeabilities with 10% m/V DMSO	41
Table 3-1. Proposed vitrification solution composition.....	51
Table 3-2. Glass Transition Temperature of CPA components.....	53
Table 3-3. Predicted T_g of vitrification solution	55
Table 3-4. Predicted T_g of vitrification solution	59
Table 3-5. Predicted T_g of vitrification solution	62
Table 3-6. Biocompatibility study of PVP and DPVP.....	67
Table 5-1. List of potential dominant mode and its resonant frequency at a rectangular cavity	83
Table 5-2. List of potential dominant mode and its resonant frequency at a rectangular cavity	84
Table 5-3. The dimension and physical properties of the resonant cavity system	95

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Chapter 1. Introduction

1.1 Cryobiology and cryopreservation

In 1949, Polge first accidentally found glycerol has a cryoprotective function to protect sperm [1]. Since then, the sub-zero temperature technologies have not only been used to kill cancer tumors with cryoablation but also to preserve the living biospecimen. With technological development in the last century, cryopreservation has been applied to numerous applications, including reproduction [2, 3], endangered species preservation [4-6], clinics [7-9], and fundamental research [10-13]. Cryopreservation first emerged as a tool in animal reproduction, aiding in variety selection and breeding of species such as cows and Kurobuta pork, particularly during outbreaks of widespread infectious diseases [14]. In addition, cryopreservation plays a crucial role in preserving germ cells of endangered animals and plant seeds within global biobanks. Currently, there are over 120 such biobanks worldwide, collaborating under the International Society for Biological and Environmental Repositories (ISBER) [4, 15]. Furthermore, ISBER launched a special interest group dedicated to cryopreservation science in 2024. In clinics, cryopreservation is an important tool in advanced cellular therapy [12, 16], blood transfusion [17, 18], and vaccination development [19]. Moreover, the preservation of sperm and oocytes benefits those nature or nurture infertility patients. In fundamental research, cryopreservation offers a long-term storage solution for living biological samples, crucial during pharmaceutical development and the study of biological systems such as organoids [20]. Although different samples may require specific cryopreservation methods and adhere to various regulations, most protocols share key steps as depicted in Figure 1-1. These steps include sample collection, addition of cryoprotective agents (CPAs), cooling/vitrification, storage, and thawing. Following thawing, the sample is evaluated before use.

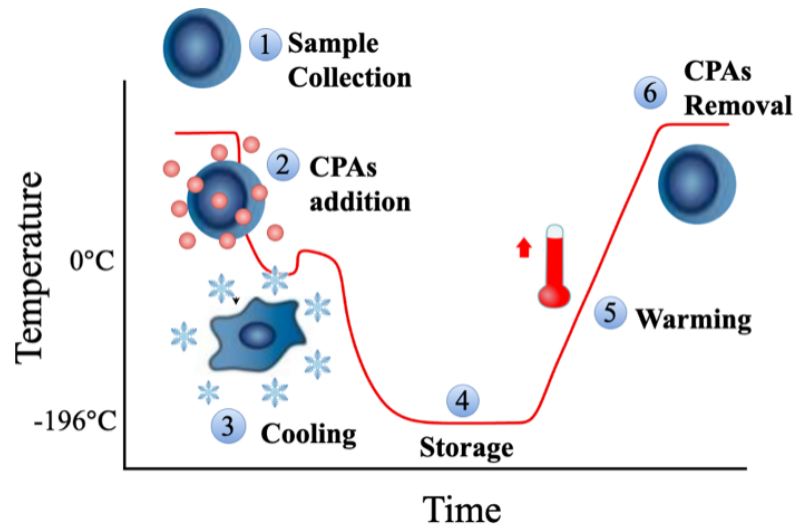


Figure 1-1. Cryopreservation standard procedures. The sample's temperature was first dropped to 0 °C for CPAs addition, and then cooling started until reaching the target temperature. Storage requires suitable moisture and a steady temperature. Then, the sample is rewarmed from ultra-low temperature to 0 °C, and CPAs are removed. Lastly, sample functionality and metabolism are evaluated before future use.

1.1.1 Slow freezing cooling

The principle of cryopreservation is to use low temperatures to reduce the biological and chemical reaction rate in biological samples to extend preservation time. In 1972, Mazur proposed the "Two-factor hypothesis" of freezing injury, inspired by research on Chinese hamster tissue [21]. Figure 1-2 presents the "Two-factor hypothesis". When cells are cooled beyond its freezing temperature, ice crystals start to form and grow in the extracellular fluid outside the cell membrane [22]. The plasma membrane restricts these ice crystals from penetrating the cells. Meanwhile, large molecules inside the membrane help keep the cytoplasm unfrozen and in a supercooled state [23]. If the cooling rate is too high, The rapid drop in temperature disrupts the supercooled state, leading to the formation of intracellular ice (IIF), which can rupture cell membranes and cause cell death [24]. Conversely, a very slow cooling rate allows ample time for water to be drawn out, mitigating intracellular ice formation. However, this slow process pushes cells to high concentrations of intracellular solutes/electrolytes, so-called "solution effect," which leads to severe dehydration.

Both IIF and solution effects are deadly to cells. Thus, cell-type-dependent optimal cooling rates are desired.

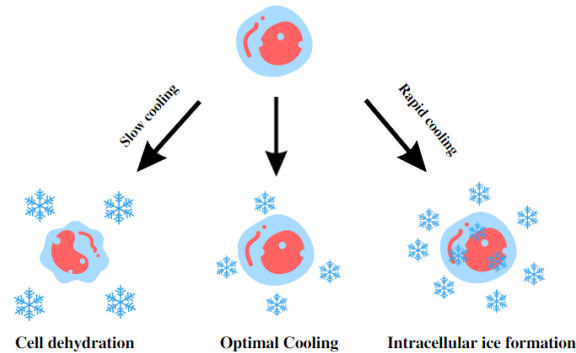


Figure 1-2. Peter Mazur’s “Two-factor” hypothesis. Cells will be dehydrated due to the solution effect under a slow cooling process. In contrast, Intracellular ice will form inside the cell, causing cell death under rapid cooling. Thus, the cell-type dependent optimal cooling rate is applied to avoid both intracellular ice formation and solution effect.

Later, scientists reported that a higher rewarming rate could increase the recovery rate of the cryopreserved samples [23, 25]. While rapid cooling leads to the formation of small intracellular ice crystals, these crystals tend to be unstable thermally. During the rewarming phase, these tiny crystals melt and aggregate since the temperature is still lower than the freezing point, so-called recrystallization. It has been shown that cells cooled at rates far beyond the optimal can survive if they are rapidly warmed, whereas slow warming leads to cell death [26].

1.1.2 Vitrification cooling

Vitrification is an alternative cooling method for forming an amorphous, gel-like state, which is commonly discussed in polymer composition studies [27-29]. Figure 1-3 shows an example of frozen and vitrified rabbit kidneys by Fahy in 1984. Since the tissue and organ contain various types of cells and each has an optimal cooling rate, vitrification addresses these challenges associated with the cryopreservation of large samples such as tissues and organs, by reaching the glass state. The minimal cooling rate necessary for this process is known as the critical cooling

rate (CCR). Vitriifying pure water or isotonic solutions requires a CCR of 10^6 °C/min, which presents significant difficulties [10]. The addition of CPAs and large molecular polymer can significantly reduce the required CCR to about 10 °C/min or less [30]. Although vitrification offers a promising solution for safe cooling, lethal ice recrystallization remains a potential risk during the thawing process. The minimum rewarming rate required to avoid this is known as the critical warming rate (CWR). Consequently, rapid rewarming is crucial in cryopreservation to ensure the viability of samples. Additionally, uniform temperature distribution during thawing is crucial for the survival of the samples [31-33]. Mechanical stress induced by high temperature gradients, particularly in large samples, can cause cracks in brittle materials [31, 34, 35]. Therefore, rapid and uniform rewarming technology is essential for successful cryopreservation, especially large-volume biological samples.



Figure 1-3. Frozen kidney vs. vitrified kidney by Gregory Fahy [27]

The expansion in clinical tests for biomedical applications revealed the limitations of current preservation technologies, only the small volumes (less than 2mL) of stem cells could be successfully cryopreserved. In the case of large systems such as bulk volumes of cells, tissues, or organs, cryopreservation often fails because of ice crystal growth within the bio-samples and

damages the biospecimen [27, 36]. However, cryopreservation still has significant needs to expand its applications. In the United States, there are almost 110K people on the national transplant waiting list because of end-stage organ diseases, including kidney, liver, lung, and heart failure in 2021. Over 40K transplantations were performed in 2020. However, there are still 17 deaths per day because of shortage of matched organs [37]. With current normothermic and hypothermic machine perfusion method, most organs can only be preserved for less than 24 hours and around 28000 of donated organs were wasted each year [38]. If this limitation has been solved by the application of organ cryopreservation, thousands of donated organs and end-stage patient life will be saved. The extended tissue or organ life can make matching, searching, shipping, and transplantation become more feasible and promising. Moreover, it also encourages the development of gene therapy and tissue engineering. Thus, there is an urgent need to scale up cryopreservation.

Overall, cryopreservation is one of the most promising methods to extend biological samples' life [39, 40]. With the development of vitrification solutions [27] and the study of critical cooling rates [16], intercellular ice formation (IIF) and lethal dehydration may be avoided [21, 29]. Nevertheless, the need for rapid and uniform rewarming technology remains critical in the field of cryopreservation. Successfully rewarming cryopreserved biological systems demands warming rates significantly higher than those of cooling, without inducing temperature gradients. This requirement presents a substantial engineering challenge in energy conversion. Consequently, there has been a surge in studies focused on highly effective energy conversion techniques for biomaterials in cryopreservation rewarming.

1.2 Current available method in rewarming

In the present literature review, various energy conversion/transfer mechanisms in cryopreservation are illustrated and compared, including thermal to thermal energy (water bath and forced air heating), mechanical to thermal energy (ultrasound heating [41, 42]), and electromagnetic to thermal energy. Electromagnetic energy can be classified as laser [43-45], electric field [46-50], and magnetic field [51-53] energy sources. In electric field heating, various applications including Joule heating, capacitor heating, antenna heating, and cavity heating have been reported, with operational frequencies ranging from Hz to MHz. Magnetic-to-thermal energy conversion primarily employs induction heating, which involves both eddy current and magnetic hysteresis effects. Fig. 1-4 illustrates this energy conversion types, and their mechanisms as applied to cryopreservation rewarming.

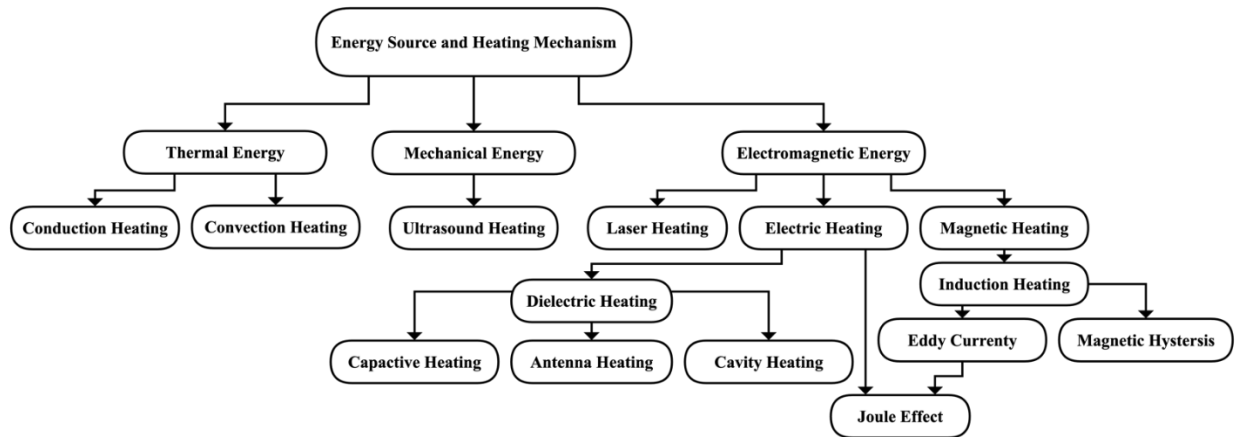


Figure 1-4. Energy conversion sources and methods in cryopreservation. Currently available rewarming technology includes three primary energy sources: thermal, mechanical, and electromagnetic. Their heating mechanism and essential applications followed each of them.

1.2.1 Conventional methods

When using thermal energy to warm cryopreserved samples, traditional rewarming methods utilize conduction or convection heat transfer mechanisms, which rely on the temperature

gradient between the heating source and the cryopreserved biomaterials. Since this temperature difference drives heat diffusion across the sample, it often results in non-uniform rewarming and thermal stress. Over the past decades, scientists have developed and refined several conduction and convection rewarming devices. These innovations aim to enhance rewarming rates, prevent ice recrystallization, achieve temperature uniformity, and minimize the risk of overheating the outer surfaces during the thawing process.

1.2.1.1 Conduction Heating

Conduction heating transfer happens when two objects have temperature gradients, and the intermediate media is solid or stationary fluid, either liquids or gases. In cryopreservation, conduction heating is also known as a water-free rewarming method. In 2002, a semi-automatic dry thawing device was developed by Saharda, Tansmed, Germany, using the two heating chambers and applied to the investigations of leukapheresis products [54]. In this application, one chamber is used to warm gel pads that fit the sample bags, and another chamber contains a heated metal platter for the sample with an infrared temperature sensor. The sample rewarming period is 3-5 minutes. However, the rewarming rate is not reported. Later, the Progenitor cell (100-140 mL) was successfully preserved and rewarmed with a dry thawing device, achieving about 2.58 °C/min and obtained a similar viability result compared to the water bath rewarming, which might be more favor to use due to the lower risk of contamination [18]. Recently, leukapheresis cells (between 60 -140 mL) were rewarmed by a controlled-rated thawing station (VIAThaw, Cytiva, Cambridge, UK), which utilized two heated metal plates setting at 34°C to warm the cryobag from the top and bottom sides [55]. Figure 1-5A represents the schematic drawing of the dry thawing device. The two-sided hot plate will heat the sample surface, and the thermal energy is deposited into the

sample inside through the conduction heat transfer. The heat flux of the dry thawing heating methods with conduction heat transfer can be described with the Fourier equation.

$$q'' = -k_x \frac{\partial T}{\partial x} - k_y \frac{\partial T}{\partial y} - k_z \frac{\partial T}{\partial z} \quad (1-1)$$

Where T is temperature, x, y, z are the three-dimension in cartesian coordinates, k_x, k_y , and k_z are the thermal conductivities in x, y, z direction.

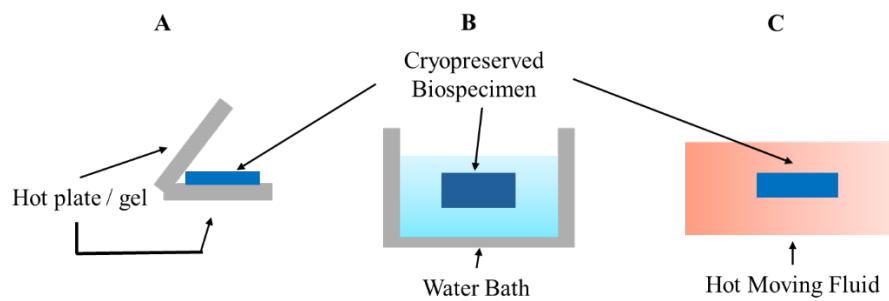


Figure 1-5. Illustration of the conventional heating method in cryopreservation.

1.2.1.2 Convection Heating

Convection heat transfer occurs when a high-temperature fluid, either liquid or gas, moves over cryopreserved samples. This process is governed by Newton's law of cooling, which states:

$$q'' = h(T_s - T_\infty) \quad (1-2)$$

Where q'' is the heat flux W/m^2 , T_s is the cryopreserved sample temperature, T_∞ is the hot fluid temperature, and h is the convective heat transfer coefficient.

The water bath serves as the gold standard in current clinic usage to rewarm low-temperature matters, which relies on convection heat transfer between liquid (mainly 37 °C water) and sample container, as shown in Figure 1-5B. For samples under the conventional cooling method, the outer layer of solid ice first melts and turns into liquid water due to the high thermal

gradient between the source and the sample. Since ice has a larger thermal conductivity (k), 8 W/m·°C [56] and less heat capacity (C), 700 J/°C·kg [57] at -193°C compared to liquid water, 0.56 W/m·°C and 4217 J/°C·kg at 0 °C [58] , both the thermal resistance and required energy in raising temperature per degree increase, which transfers the sample from a good thermal conductor to a poor thermal conductor and reduces the thawing rate. For successfully vitrified samples, water baths are unable to achieve the CWR necessary to prevent ice recrystallization during thawing [59]. In both scenarios, high temperature gradients between the outer surface and the core generate substantial thermal stress, which can lead to cracking [32]. Therefore, although the water bath is the cheapest and most convenient thawing approach, it still has some unavoidable and fatal challenges [60]. Table 1-1 illustrates some represented rewarming experiments with water bath for various sample types and volumes. The small sample has a higher rewarming rate and less temperature gradient caused by spatial effect. When the sample size increases substantially, the thawing rate is significantly reduced. Overall, the water bath is only suitable for the millimeter-level and non-vitrified samples.

TABLE 1-1. WATER BATH REWARMING EXPERIMENTS OF CRYOPRESERVED SAMPLES

Sample Name	Total Volume (mL)	Water Bath Temperature (°C)	Shaking	Rewarming Rate (°C/min)	Recovery Rate (%)
Rat Islet slice[61]	0.1	37	-	> 1000	79
Bovine embryo[25]	0.25	35	×	> 1000	42.1
Testicular interstitial cell[62]	1	37	√	20	65.3 ± 2.1
Rabbit Carotid Artery[63]	10	37	×	130	71
DPVP Solution[48]	25	37	√	48.2	-

DPVP is a vitrification solution contains 41% (v/v) dimethyl sulfoxide (Sigma Aldrich, St. Louis, MO) and 6% (w/v) polyvinylpyrrolidone (Sigma-Aldrich) in phosphate buffered saline solution (Sigma Aldrich). – means information is not mentioned in cited references.

Another convection heat transfer in practice relies on the forced moving hot air as a heating fluid, shown in Fig. 1-5C. S. Hochi et al. compared the water bath and natural air convection heat transfer results with morulae and blastocysts [25]. In this work, the air rewarming rate is less than 250 °C/min while the water bath warming rate is larger than 1000 °C/min for 0.25 mL sample. Consequently, slow warming appears to cause injuries to morulae and blastocysts by comparing the hatching results after cryopreservation. For the 25 mL sample size, Shen presents that air rewarming is not as good as a water bath in the rewarming rate but better in temperature uniformity [48]. However, air rewarming is not commonly applied to the thawing of cryopreserved samples due to the insufficient rewarming rate.

1.2.2 High intensity focus ultrasound

The ability of ultrasound to safely penetrate deep tissues makes it an attractive option for the noninvasive treatment of diseases and internal injuries [64]. Ultrasound heating is converting the mechanical energy of ultrasound waves into heat, governed by the following equation [65]:

$$Q = \alpha I = \frac{\alpha p^2}{\rho c} \quad (1-3)$$

Where the Q is the heat generation rate per unit volume (W/m^3), α is the acoustic absorption coefficient of the medium ($\text{Np}/\text{m}/\text{MHz}$), I is the ultrasound intensity (W/m^2), p is complex pressure profile [Pa], ρ is density of sample [kg/m^3] and c is sound velocity.

By using a focused ultrasonic beam, high-density mechanical energy is delivered to a small region of tissue with little or no effect on the surrounding areas. High-intensity focused ultrasound (HIFU) is widely used in thermal ablation of cancer treatment. HIFU heating using commercial transducers successfully increase the temperature of the tumor sample within 1 mm diameter in

1200 °C/min [66]. Olmo et al. took the benefit into the cryopreservation field to elevate cryopreserved samples' temperature from -150 °C [41]. With the study of different frequencies and power input, 200 °C/min is achieved for organ ($\sim 3 \text{ cm}^3$) when applying 75 W and 300 kHz from simulation results. Xu et al. first demonstrated the rewarming rate in an experiment in 2022 and accomplished a maximum rewarming rate of 57°C/ min (mean 23°C/min) for 2mL cryovial from -20°C to 5°C using 444 kHz ultrasound [42]. Although the simulation result presents rapid rewarming rates, it still lacks evidence that can be realized in the experiment until 2023. In 2023, Alcalá et al. utilized HIFU to successfully rewarm *Caenorhabditis elegans* nematodes cryopreserved from -80 °C [65]. This method effectively addresses recrystallization challenges by harnessing ultrasound's inherent attenuation properties, reducing the risk of thermal runaway caused by electromagnetic heating described in the following section. Unlike electromagnetic rewarming, which is limited by coil positioning, HIFU provides superior flexibility and precision. Building on this progress, Encabo et al. achieved the recovery of mouse hearts from cold preservation at -6 °C using the HIFU technique in 2024 [67]. These advancements highlight HIFU's potential as a promising solution for mitigating recrystallization-related issues in rewarming process of cryopreservation. However, the ultrasound propagation is influenced by wavelength, pressure, velocity, power, intensity, sample volume, and material property such as density and acoustic impedance. Thus, the HIFU is still limited in small sample size results from low penetration depth since the ultrasound intensity decreases significantly when the material has high acoustic impedance such as bone [68]. In addition, heating uniformity still needs to be evaluated to understand the thermal stress during HIFU heating method. Lastly, HIFU is also limited by ultrasound imaging technology, such as acoustic shadowing, reverberation, and refraction.

1.2.3 Electromagnetic rewarming theory

1.2.3.1 Plasmonic Laser Heating

Laser heating increases the temperature of cryopreserved samples by absorbing electromagnetic energy in the optical wavelength range. Figure 1-6 demonstrates the setup of the laser heating and the light scattering effect produced by the nanorod. The composition of the laser heating setup includes near-infrared (NIR) lasers, a collimator, lenses, a magnetic stage, and a sample cuvette, shown Figure 1-6. Three beam splitters and four mirrors are included to further enhance the heating uniformity. Titanium nitride (TiN) and gold (Au) are the two most commonly used plasmonic nanomaterials dispersed into CPA to enhance energy conversion from optical radiation to thermal energy [45]. Daly et al. and Khosla et al. demonstrated that the pulsed laser (1064nm) gold nanoparticles (GNs) heating method could raise the temperature of vitrified sample droplets at a rate larger than 1.0×10^7 °C/min for microliter samples [69, 70]. In 2021, Zhan et al. further proved that the rewarming rate of laser heating for microliter scale samples is 400 times greater than the traditional convective method (water bath) [44]. Compared with GNs, TiN nanoparticles (NPs) are not only less expensive but also have high thermal stability. Meantime, TiN NPs demonstrated higher absorption but less scattering than Au nanorods, which leads to TiN clusters and a higher heating rate compared with Au nanorods (GNRs). In 2022, Alvarez et al. ascertained that TiN NPs make improvements to heat rate, thermal stability, and temperature uniformity than GNs [45]. Additionally, a 1µL microdroplet was studied using 1064nm pulsed laser heating, and devitrification was not observed. Although laser heating achieves a fast-thawing rate, it is still limited to small-size sample because of the laser focus size. In addition, the high concentration of GNRs or TiN NPs brings high costs and unknown toxicity.

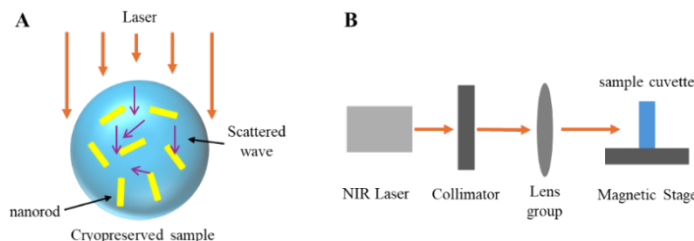


Figure 1-6. Heating mechanism of laser rewarming and its setup. A shows the mechanism of laser rewarming with nanorod/nanoparticle infused into the cryopreserved sample. The laser projects to the cryopreserved sample, such as cells, and dispersed by the nanorod across the sample volume. The scattered wave heats the cell uniformly. B shows the general setup of the laser rewarming application

1.2.3.2 Electric Energy Source

The electric-to-thermal energy conversion heating method incorporates principles from both low-frequency conventional electric circuit theory and high-frequency electromagnetic wave theory. The former relies on electric resistance induced by ionic movement (Joule effect), while the latter utilizes the dielectric effect, which stems from the polarization of dipole molecules such as water and DMSO, as illustrated in Figure 1-9. Figure 1-7 illustrates the oscillation of particles at various frequencies. As higher frequencies are selected, smaller masses of matter are excited due to the interplay between relaxation time, mass, and frequency. Dielectric heating capitalizes on this principle by utilizing the rotation of dipolar molecules in an alternating electric field, thereby generating volumetric heat through molecular friction.

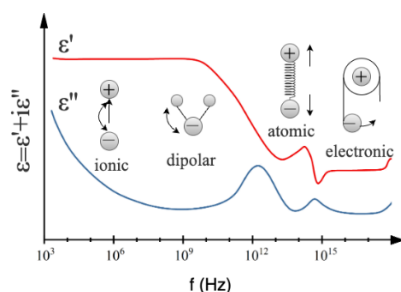


Figure 1-7. Dielectric property resonant status of material versus frequency relationship. With increasing frequency, the wavelength becomes smaller, and the oscillated mass becomes smaller, from ionic to electronic.

I) Joule Heating

Joule heating, the physical effect, is widely employed in medical treatments, including radiofrequency ablation for cancer. [71, 72]. It occurs when the current passing through a resistive conductor produces heat. Zhan et al. firstly proposed a joule-heating-based platform to rewarm the vitrified biosystems [73], including adherent cells ($\sim 4\ \mu\text{m}$), *Drosophila melanogaster* embryos ($\sim 50\ \mu\text{m}$), and kidney slices ($\sim 1.2\ \text{mm}$) were placed on the electrical conductor with pulsed voltage input (up to 350 V). With tunable voltage pulse widths, they achieved a rewarming rate of $2.4 \times 10^6\ ^\circ\text{C}/\text{min}$ from $-196\ ^\circ\text{C}$ to $7.6\ ^\circ\text{C}$. Later, Han developed a joule heating-based electric heating chip and retrieved 97.2% viability of human lung adenocarcinoma cells and human mesenchymal stem cells (MSCs) with 93.18% [59]. With about 185W (4A current and $11.6\ \Omega$ heating sheet resistance), they achieved $2300\ ^\circ\text{C}/\text{min}$ for 2.8mL vitrified solution.

Figure 1-8 illustrates two common structures of Joule heating: a flat sheet (Figure 1-8, left) and a sandwich structure conductor (Figure 1-8, right). Both structures employ electric current and its inherent resistance to generate heat, which then diffuses from the conductor to the samples. Joule heating offers the advantage of a simple setup and mechanism, similar to that of a resistance heater. However, precise control of the system—considering factors such as pulse width, voltage magnitude, current magnitude, operation time, and heat diffusion time for specific samples—is crucial to ensure the rewarming rate exceeds the CWR and to prevent overheating at the interface between the sample and conductors. Additionally, as the size and thickness of biospecimens, such as fat tissue and organs, increase, achieving uniform heating becomes more challenging. The contact layer between the sample and the conductor consistently reaches the highest temperature, posing a significant risk for larger samples, where such localized overheating can be detrimental. Joule heating is governed by follow equation:

$$q' = I^2 R \quad (1-4)$$

Where q' is the heat generation per unit length, I is the current in A, and R is the electric resistance in Ω .

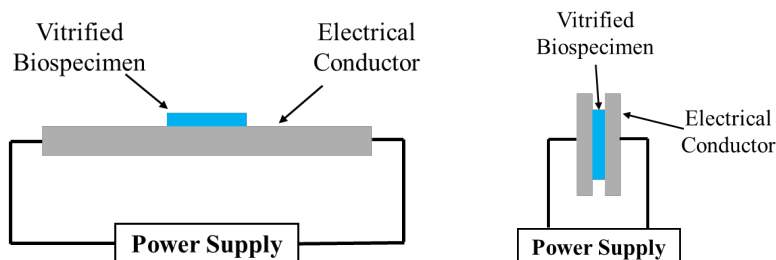


Figure 1-8. Schematic diagram of the joule heating setup. Left figure represents a sample directly placed on the flat sheet electrical conductor. The sample will be heated by joule heating after the power is applied to the electrical conductor and the bottom layer of the sample always heated first; Right figure shows the sandwich structure for tissue slices.

II) Dielectric Heating

The electromagnetic wave heating effect was first discovered by Percy Spencer in 1945 [74]. Later, the conventional microwave was invented in 1947 and became more common in the 1970s [74]. The study of microwave thawing in cryopreservation began in the 1960s [75]. Dipolar molecules, like water illustrated in Figure 1-9 (left), exhibit an internal charge difference owing to the high electronegativity of the oxygen atom. Consequently, water molecules align with the direction of the electric field, generating substantial heat through friction when subjected to an alternating electric field. This mechanism, shared by substances such as DMSO and glycerol, is known as dielectric heating.

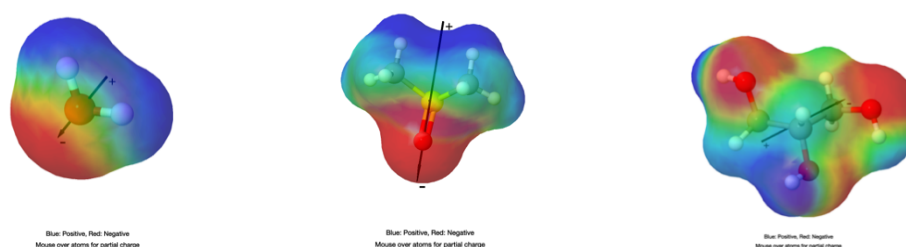


Figure 1-9. Molecular structure of water (left), DMSO (middle), and glycerol (right). The red region shows the negative charge, while the blue shows the positive charge. The black arrow line represents the direction of the electric potential.

The dielectric heating is governed by the following equation:

$$Q = \omega \cdot \varepsilon''_{eff} \cdot \varepsilon_0 \cdot E^2 \quad (1-5)$$

Where Q is the heat generation rate per unit volume, W/m^3 , ω is the angular frequency of the exciting radiation, rad/s, which equals to $2\pi f$ and f is the applied frequency, ε_0 is the permittivity of free space and E the electric field strength. ε''_{eff} is the effective relative permittivity loss, which can be classified as dielectric, ε''_d , and conductivity contribution, ε''_σ , expressed by the following equation:

$$\varepsilon''_r = \varepsilon''_d + \varepsilon''_\sigma = \varepsilon''_d + \frac{\sigma}{\omega \varepsilon_0} \quad (1-6)$$

Where ε''_d is the imaginary part of the complex relative permittivity of the absorbing material, describing the ability to convert electromagnetic field energy into heat; σ is the electric conductivity. For non-conductive material, $\varepsilon''_{eff} = \varepsilon''_d$, for high conductive material, $\varepsilon''_{eff} \approx \varepsilon''_\sigma$.

In the following section, current available dielectric heating applications in cryopreservation rewarming fields will be discussed, whose all follow the above theories, including a) capacitor heating, b) antenna heating, c) multi-mode cavity heating, d) single mode resonant cavity heating.

III) Capacitor Heating

Conventionally, capacitor heating devices consist of two electrodes, a coupling coil, a power supply, and a temperature sensor. In 1964, Lehr [76] first proposed the concept of using capacitor heating for organ preservation, including concentric cylindrical electrode configuration, parallel plate electrode configuration, and multiple electrode configuration (Figure 1-10) at 27 MHz and 440 W. Concentric cylindrical electrode configuration (Figure 1-10A) only works for small samples, while the larger masses could not be warmed faster than 10 °C/min without

overheating the significant amounts of tissue in the vicinity of the internally cooled central electrode, which is similar to the radiofrequency ablation [72], a minimally invasive cancer treatment method. Figure 1-10C shows the six-electrode organ heating system, which allows the heat of the organ in three arbitrary areas (upper, lower, and middle). However, the overheating of significant amounts of tissue cannot be avoid when thawing rate is greater than $10\text{ }^{\circ}\text{C}/\text{min}$ because of inadequate methods of temperature monitoring due to the difficulty of placement and unusable thermocouples in the electric field (E). Currently, no impedance matching was considered in the current work.

Recently, Wowk continued to work on parallel capacitor heating (Figure 1-10B) with the improvement of impedance matching [77]. A rabbit kidney perfused with M22 vitrification solution at 50 mL total volume was vitrified and thawing at 27 MHz constant frequency with 500 W power. The peak rewarming rate near $150\text{ }^{\circ}\text{C}/\text{min}$ was achieved at $-70\text{ }^{\circ}\text{C}$, and the average rewarming rate from $-100\text{ }^{\circ}\text{C}$ to $-30\text{ }^{\circ}\text{C}$ is about $55\text{ }^{\circ}\text{C}/\text{min}$. This system involves a forward/reverse power meter, parallel and series variable capacitors, two couple coils and variable attenuator. Forward/reverse power meter is used to monitor the ratio of forward and reflected power. Impedance matching was achieved by manipulating the 0-20 dB variable attenuator to maintain a constant field intensity.

Thus, the parallel capacitor is a more widespread configuration for study. Both joule heating and parallel capacitor heating utilize two flat plate electrodes/conductors. The key differences between those two are frequency utilization and heating mechanism. Thus, the sample doesn't require contact with the electrode and results in volumetric heating with an alternative E field. The advantages of this system are high penetration depth, a high absorption rate of M22 with designed 27 MHz frequency signal input, and impedance matching control by adjusting the

variable capacitor. However, this system also holds a couple of limitations, including 1) insufficient rewarming below -100°C mentioned in Wowk's work [77], 2) an increase of energy loss when parallel electrode distance increases with increasing sample thickness, 3) limited power allowance to avoid voltage breakdown, 4) safety risk due to the high voltage difference between two electrodes, 5) low system quality factor Q represents low efficiency.

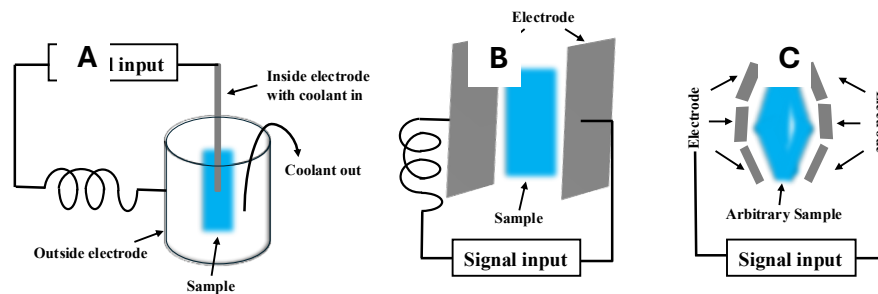


Figure 1-10. Configuration of capacitor heating. (A) Concentric cylindrical electrode configuration, (B) parallel plate electrode configuration, (C) multiple electrode configuration

IV) Antenna Heating

Antenna heating heats the cryopreserved samples directly with the incident power from the antenna. Although the E and magnetic (H) fields will be excited from the antenna, biological samples are barely able to absorb the H field energy without doping magnetic matters such as ferrous electro-seeds or magnetic nanoparticles, which will be described in induction/nanorewarming. Burdette and his colleagues [78] designed a single (2450 MHz) and double frequency (2450 MHz + 7MHz) horn-shaped antenna to heat the cryopreserved kidney of rabbit (N = 74) and canine (N = 27). The 7 MHz antenna is placed orthogonal to the 2450 MHz source to enhance the uniformity for larger samples, such as canine kidneys, by taking advantage of the larger penetration depth and specific local doping ferrous electro-seed. Later, 918MHz was applied by the same group but achieved better temperature uniformity for 27 canine kidneys with a single frequency horn-shaped antenna heating system and no additive required. Unfortunately,

no vitrification and transplantation were performed. Recently, a hybrid warming method combining traditional convective heating [79], water bath, and the helical antenna has been studied in a 1.8 mL cryovial shown in Figure 1-11 (right), which includes a signal input from the coaxial feeder, helical antenna excites axial E field and water bath. Although a high rewarming rate was achieved, 100.05 °C/min, with 3 W signal input, the studied sample size was still limited, less than 1.8 mL, due to the space taken by the helical antenna.

Both horn and helical antenna heating offer distinct advantages. Horn antenna heating [80] can handle larger power inputs without risking damage to the antenna, unlike helical antennas, which may sustain damage from reflected power when the input power is significantly increased. Additionally, horn antennas are suitable for heating cell suspensions, tissues, and various organ types. Conversely, inserting helical or other uniquely shaped antennas directly into organs can be challenging without compromising the structural integrity of the samples. Nevertheless, a hybrid heating approach combining helical antennas with a water bath provides a cost-effective and sufficient method for heating cell suspensions, with potential for scaling up sample sizes.

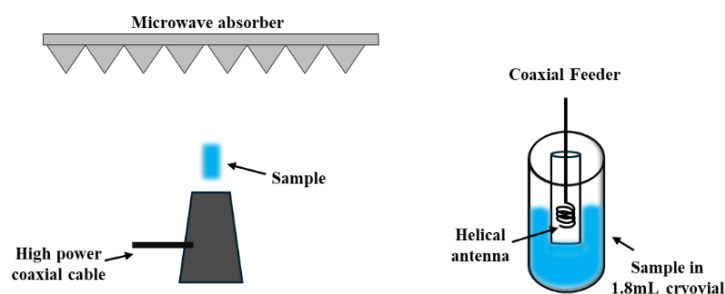


Figure 1-11. Schematic drawing of the antenna heating. The left figure shows the horn antenna with microwave absorber. Microwave absorber is used to avoid the reflected wave disrupting the E field. (Need to be embedded to the horn antenna. Right figure shows the hybrid helical antenna heating method with water bath. (need to draw water bath outside the sample holder)

V) Multimode Cavity Heating

Ketterer et al., Guttman et al., Rachman MJ et al., and Pegg et al. successively reported using microwave thawing for canine kidneys at frequency of 2.45 GHz [75, 81, 82]. However, the limited penetration depth associated with high frequencies results in non-uniform heating across the radius of biological samples. Additionally, the non-uniform distribution of the electric field can lead to 'thermal runaway,' a problem exacerbated as the ability of the material to absorb electrical field energy increases with rising temperature. Subsequently, the multi-mode cavity was designed to enhance the uniformity of electrical wave peaks within the chamber. However, real-time analysis and control of the multi-mode cavity system are challenging due to the material's permittivity, which is complex and varies with temperature and frequency [83]. As the temperature increases, changes in the real part of the permittivity of cryopreserved samples lead to off-resonance and shifts in the peak electric field, which constrain further development and application of this technology.

VI) Single-mode Resonant Cavity Heating

Due to the difficulty described above in a multi-mode cavity or commercial microwave oven, a single-mode resonant cavity seems more promising in providing not only high intensity and uniform E fields but also ease of prediction and control [84]. Two common configurations of single-mode resonant cavities are cylindrical and rectangular. Considering the future application of the organ, a rectangular cavity was developed with 434 MHz by Luo in 2006 to ensure the size allowance, penetration depth, and easy manufacturing ability as the cylindrically shaped cavity is smaller than a rectangular cavity at the same frequency.

Overall, scientists paid lots of attention to electric heating since the high-power intensity ability for the range low and high frequency. Multiple applications have been developed, and each

of them has its own pros and cons. However, some common requirements and challenges for electric heating are the need to control the power feed width band, frequency, sample size, and impedance/resonant tuning to achieve a constant high rewarming rate.

1.2.3.3 Magnetic Energy Source

Similar to electric field heating, magnetic heating also performs volumetric heating of the reactive material, so-called induction heating. Induction heating relies on the eddy current or hysteresis effect. No matter what type of mechanism is used in magnetic heating, the thermal seed is required for cryopreservation, such as nanoparticles, metal mesh, metal foil, metal foam, etc. There are a couple of other effects of magnetic heating mechanics, such as Néel Relaxation and Brownian Relaxation, but they have not been applied to cryopreservation yet.

I) Eddy current effect

Eddy current happens when a tangential magnetic field encounters the conductive material surface. Then, an eddy current is produced on the conductive surface, and the joule heating effect is involved. Figure 1-12 represents the eddy current under a heating coil, which can be governed as equation below:

$$W_{diss} = \frac{R_s}{2} \int_{wall} |H_{tan}|^2 ds \quad (1-7)$$

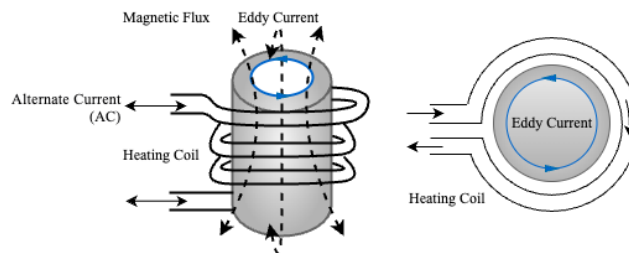


Figure 1-12. Eddy Current illustration. The left figure shows the 3D drawing of the eddy current under and alternative current produced magnetic field. With the changing direction of the current, the magnetic flux is created

and located at the center of the coil. The right figure shows the top cross-section. The eddy current occurs when the magnetic field is tangential to the conductive material surface.

Manuchehrabadi [52] studied Eddy current-induced rewarming for cell suspension by applying copper foam, aluminum foil, and nitinol mesh. It achieved 1200 °C/min at 1.8 mL vitrified sample in cryovial with RF power of 1kW. To successfully convert magnetic field energy to eddy current, the foil thickness should be 4 times the penetration depth of the aluminum foil, which depends on the surface properties and applied frequency.

II) Hysteresis effect/nano-rewarming

Hysteresis refers to a broad class of phenomena in which the response of a system depends not only on its current state but also on its history. This effect manifests in many different forms across disciplines, including mechanical, thermal, electrical, and magnetic systems. Among these, magnetic hysteresis is one of the most well-known, describing the lag between changes in magnetization of a material and the external magnetic field applied to it. This delayed response results in characteristic hysteresis loops, which are widely studied in physics and engineering for their implications in energy loss, memory effects, and material behavior. The hysteresis effect depends on the stored and released magnetic field (H) energy in the magnetic materials, such as magnetic nanoparticles, which are popularly used in nano-rewarming technology. When the positive magnetizing force is applied, the magnetic material starts from 0 to A (situation) under the dashed line. Then, when the field is removed or switched to another direction, the material follows the line from ABCD in Figure 1-13A. Then, the magnetic field direction switches again, and the curve follows DEFA. Hysteresis loss effect occurs when a magnetic material is subjected to an alternating magnetic field, causing the magnetic dipoles to oscillate and change polarity with each cycle. This oscillation leads to considerable internal friction and results in heating within the

material [85]. This enclosed area is the energy loss for the magnetic hysteresis effect shown in Figure 1-13A. The average heat generation q is expressed as:

$$q = P_v = k \cdot f^a \cdot B^b \quad (1-8)$$

Where P_v is the time average power loss per unit volume mW/cm^3 , f is the frequency in kHz, and B is the peak magnetic flux density; k , a and b are called the Steinmetz coefficients, which are materials parameters generally found empirically from the materials B-H hysteresis curve fitting.

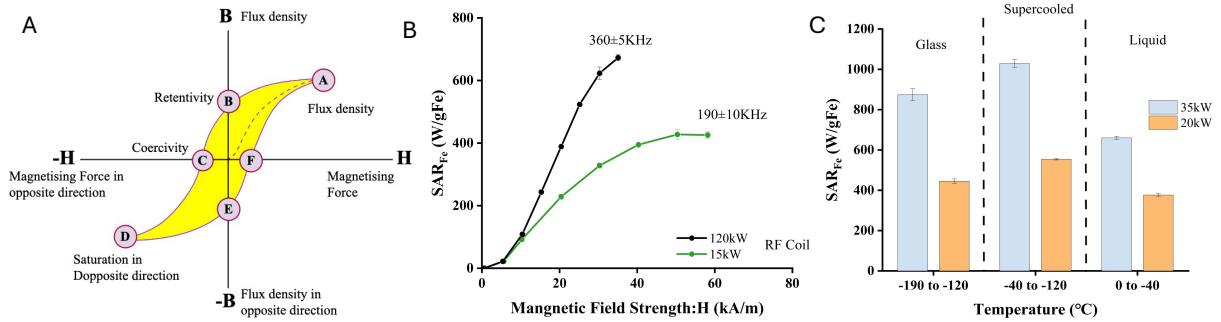


Figure 1-13. Magnetic Hysteresis Effect and Nanorewarming Results. (A) Illustration of the magnetic hysteresis curve (B-H). (B) Magnetic field strength to the specific absorption rate (SAR) in 360 and 190 KHz at 120 kW and 15 kW. The SAR expresses the energy absorption rate of an electromagnetic field impinging on human body tissue. (C) SAR of the magnetic nanoparticle in various temperatures and magnetic field strengths.

Based on the magnetic hysteresis effect, a research group at the University of Minnesota initiated the radiofrequency (RF) nanowarming technology, which depends on the excitation of magnetic nanoparticles (SNPs) by the magnetic field. Etheridge et al. first presented proof of concept RF nanowarming to achieve $200\text{ }^{\circ}\text{C}/\text{min}$ for 1 mL CPA solution containing 10 mg Fe/ml samples when supplying 1 kW power [51]. Following this study, Manuchehrabadi et al. scaled up the RF nanowarming system to 80 mL physical system[52]. They demonstrated that both 50- and 80-mL rewarming rates are at $120\text{ }^{\circ}\text{C}/\text{min}$. In 2021, Sharma et al. successfully vitrified rat kidneys and reached a mean thawing rate of $63.7\text{ }^{\circ}\text{C}/\text{min}$ [53]. Long-term vitrified rat kidneys were successfully thawing and translated by the same group in 2023 [86] and they are exploring the

vitricification and nanowarming in human size organ currently [87]. In addition, they are actively working on the nanoparticles development [88, 89] and the CPAs loading protocol [90]. Nanowarming, which leverages nanomaterials and the magnetic hysteresis effect, appears to be a promising method for scaling up cryopreservation while maintaining rapid and uniform heating.

1.3 Challenges in rewarming

In summary, traditional water baths cannot promise rapid and uniform rewarming to avoid lethal ice recrystallization and thermal stress for large samples such as tissues and organs. Laser and ultrasound heating are also limited to small-scale samples. Thus, dielectric and induction heating mechanisms become more attractive due to its large-size volumetric heating results. Each of them has its own advantages, limitations, and potential improvements discussed below.

1) Power usage. Dielectric heating consumes significantly less power than induction heating methods, which rely on eddy current and hysteresis effects [50]. Recent research demonstrates this efficiency gap, with nanowarming requiring a 120 kW power source while comparable dielectric heating needs only 500 W, highlighting the poor energy conversion between magnetic field energy and thermal energy. This inefficiency creates an additional challenge: RF nanowarming systems require extensive cooling infrastructure to prevent coil overheating. On the other hand, dielectric heating faces its own limitation—despite its energy efficiency, the dielectric signal generators and power amplifiers are costlier than induction coil systems, which are already well-established in industrial applications. This cost disparity ultimately constrains the scalability of dielectric heating technologies.

2) Additives. Benefiting from dielectric heating of the dipolar molecule, NPs are not required since water/CPAs are the energy absorption media of dielectric heating, while the

nanoparticles/seeds are necessary in nanowarming. In addition, biocompatibility needs to be considered as additives.

3) Control Theory. Dielectric heating demands sophisticated control to satisfy impedance-matching requirements throughout the thawing process. SMER (single-mode electromagnetic radiation) heating offers considerable advantages in field prediction and control due to the presence of only one mode. In contrast, induction heating does not face this limitation as it operates as an open system.

4) Thermal runaway. Both dielectric and induction heating methods must carefully manage thermal runaway issues. This challenge arises from the dependency of thermal runaway on the material properties, making it a critical concern for both heating techniques.

5) Hysteresis effect. Figures 1-13B and C describe the specific absorption rate (SAR) of nanoparticles in various frequencies and magnetic field strengths. The slope of SAR decreases as the magnetic field increases for both the 360 kHz (red) and 190 kHz (blue), and the higher frequency significantly increases the SAR when the same magnetic field is applied, which might result from the relative permeability change by frequency. The magnetic hysteresis effect promises the rewarming performance at the low temperature region, lower than -100°C . This observation is consistent with the hysteresis effect diagram presented in Figure 1-13A, where a higher magnetic field strength results in only a small increase when closing to the boundary area, indicating less efficiency. In addition, there is a maximum SAR in their system, indicated by a red line around 50 kA/m, which likely reaches the saturation point of its magnetic nanoparticles. The dielectric heating using 27MHz is lack of the ability rewarm sample lower than -100°C [77]. For dielectric heating, the hysteresis effect happens on ferroelectric materials, one kind of dielectric material,

which might be useful for enhancing the rewarming performance, but the solubility is a problem since most of them don't dissolve into water.

6) Cost-effective. Both SMER and nanowarming technology utilize radiofrequency electromagnetic waves, though with distinct implementations. SMER employs significantly higher frequencies in the MHz range, which increases equipment costs. Conversely, while nanowarming operates at lower frequencies, it requires high-power amplifiers and substantial cooling systems that similarly elevate equipment expenses. This makes a direct comparison of capital equipment costs challenging. However, the operation and maintenance cost in potential clinical application tend to be low since the low power used in SMER technology.

7) Clinical Operability. Since the end-users of cryopreservation rewarming devices will be medical staff who may lack physical and engineering backgrounds, clinical implementation requires user-friendly design. Both technologies should feature single-button operation and automatic shutdown capabilities to ensure ease of use. A key distinction lies in their physical setup: SMER utilizes a contained cavity system, while nanowarming employs an open RF coil that requires dedicated space similar to MRI facilities. Additionally, nanowarming's substantial power requirements may necessitate new hospital infrastructure, potentially complicating installation and increasing implementation costs.

1.4 Dissertation outline

The structure of this dissertation is organized as follows.

Chapter 2 shows the development of a 3-in-1 cell processing system and optimization of the cell concentration and cell type-dependent protocols for CPA addition/removal. The system prototype has been built and used to validate the numerical simulation. Later the simulation serves as a guide for selecting the optimal dilution flow rate and the initial osmolarity.

In chapter 3, A new verification strategy was developed by systematically considering osmotic injury, toxicity, vitrification tendency, and dielectric properties. The newly formulated cryoprotective agents (CPAs) reduce the concentration of dimethyl sulfoxide (DMSO), which is known to damage biological samples due to its high toxicity and osmolarity at elevated concentrations. To ensure sufficient vitrification tendency, the formulation compensates by increasing the concentration of polyvinylpyrrolidone (PVP).

In chapter 4, we conducted an experiment with the new proposed vitrification added to Jurkat cell sample. 4 mL of cell-CPA mixed solution was directly plugged into the solution and later rewarmed by water bath. The recovery rate and proliferation were tested after the rewarming.

In chapter 5, we study broadband frequency cavity design including the resonant cavity and reflected cavity. Later the 915 MHz resonant cavity was prototyped and equipped with solid state generator to achieve high input power. In addition, an auto tracking system was developed to track the resonant frequency during the rewarming process to address the dielectric property change of material with increasing temperature.

In chapter 6, Vitrification solutions (20 mL) containing varying combinations of DMSO and different molecular weights of PVP were rewarmed from -150°C using a 434 MHz cavity, while smaller volumes (4 mL) were rewarmed using a 915 MHz cavity. Both cavities utilized an auto-tracking, single-resonant mode at various power levels.

In chapter 7, different CPA solutions and cell volumes were evaluated using an electromagnetic resonance rewarming system operating at 434 MHz, compared against a conventional water bath method (the current clinical standard). Samples were rewarmed from

storage conditions of -80°C freezer and liquid nitrogen (LN_2). Following thawing and CPA dilution, cell recovery rates and metabolic activity were assessed.

Chapter 8 presents the experimental results obtained from mouse kidney slices. The slices were cooled to vitrification using a programmable controlled-rate freezer and subsequently rewarmed via a water bath and an electromagnetic resonance rewarming system. Following rewarming, histological analysis and metabolic activity assessments were conducted.

The final chapter (Chapter 9) addresses limitations in the current system and explores potential avenues for improvement. Building upon these findings, I propose a preliminary design for an enhanced system, focusing on improved heating performance and incorporating an additional monitoring system to further mitigate thermal runaway risks.

Chapter 2. DEVELOPMENT OF A 3-IN-1 MULTIFUNCTIONAL CELL PROCESSING SYSTEM AND OPTIMIZATION OF CELL TYPE-DEPENDENT PROTOCOLS FOR CPA ADDITION/REMOVAL

2.1 Introduction

Cryopreservation is aimed at preserving biological samples, including cells, tissues, and organs, at low temperatures for long periods (usually at $-80\text{ }^{\circ}\text{C}$ or $-196\text{ }^{\circ}\text{C}$). However, such biological samples can be damaged during the cooling or rewarming process by cryoinjuries, particularly due to intracellular ice formation (IIF) [21]. To avoid cryoinjury caused by IIF, cryoprotective agents (CPAs) should be added to the samples before cooling. Generally, two types of CPAs are utilized in cryopreservation, i.e., permeating and non-permeating CPAs. The permeating CPAs have the ability to transport through cell membranes into cells, while the non-permeating CPAs do not. After the thawing of cryopreserved samples, CPAs need to be removed due to their toxicity, which may increase as temperature rises [91]. The conventional CPA removal method is dilution-centrifugation with isotonic or hypertonic solutions, which utilizes a centrifuge to separate extracellular solution and cells based on relative centrifugal force (RCF). Subsequently, the supernatant is replaced with the desired CPA-free medium for future applications. Despite its widespread use, this method has inherent drawbacks or potential issues such as cell clumping and loss, stem cell activation, contamination, and the need for well-trained personnel and intensive labor work [11, 36, 92]. Besides, those CPA removal protocols lack the consideration of cell type-specific properties (for example, cell membrane permeability to water and CPAs), which may lead to suboptimal procedures for various cell types.

Alternative techniques may be worth exploring to address these challenges [93, 94]. In the past two decades, several non-centrifugation methods have been employed for the addition and removal of CPAs. One notable approach was introduced by Arnaud [95], who pioneered the use of hollow fiber membranes for removing dimethyl sulfoxide (DMSO), the most used CPA, from platelet concentrate. Then, dialysis was initiated for the removal of CPAs, similar to kidney dialysis, requiring one inlet and one outlet for both the blood and dialysate sides. Later ultrafiltration was widely applied since it is more efficient. During ultrafiltration, no dialysate is needed and only 1 outlet is presented in the dialysate, which is connected to the pump that generates pressure difference across the hollow fiber membranes to achieve convective mass transfer of CPAs. Those innovative techniques showcased the potential for advancements in cryopreservation methodologies. Theoretical and experimental investigations of CPA removal from cryopreserved blood with hollow fibers were performed and published by Ding et al. [93, 95, 96]. The dilution-filtration method was then further developed by Zhou et al., Qiao et al., and Liu et al. [97-99] with a modified dilution method and removal protocol. Zhou et al. also developed microfiltration-based sequential perfusion in CPA loading and unloading. Microfluidic technology was also employed to achieve CPA loading and unloading [100, 101], however, it is limited to the usage of small-volume cell suspensions (e.g., several mL).

Although previous pioneers have made notable contributions towards recovering valuable cells through various CPA removal methods, the current dilution-centrifugation and dilution-filtration methods ignore the cell type-dependent mass transfer behaviors during the CPA addition and removal, which are determined by the cryobiological characteristics such as cell membrane permeability to water (L_p) and to permeating CPAs (P_s). Mass transfer dynamics, crucially involving the transmembrane movement of water and CPAs, play a pivotal role in dictating several

fundamental phenomena observed during the cryopreservation process. These dynamics directly influence the volume changes in cells, the formation of IIF, and the cells' osmotic responses. Thus, an optimal CPA addition and removal protocol tailored to each specific cell type is desired to reduce cell damage. However, there is still a lack of a cell type-specific CPA addition/removal protocol taking into account the cell membrane permeability to both water and CPAs.

Concentration of cell suspensions (i.e., cell suspension volume reduction) is often needed either in cryopreservation or advanced therapy medicine product (ATMP) manufacturing processes. In the realm of cryopreservation, handling large sample volumes presents distinct challenges including the utilization of substantial quantities of CPAs and complication of the achievement in optimal cooling and rewarming procedures. Concentrating the cells prior to cryopreservation helps reduce the volume of cryopreserved samples and the corresponding required quantity of CPAs. Achieving successful commercialization of cell therapies necessitates meticulous planning and careful deliberation regarding product quality, manufacturing process cost, scalability, shipping, and storage [102, 103]. ATMP manufacturing process, generally includes cell collection, washing, activation, gene delivery, expansion, and formulation/filling [104, 105]. For example, the CAR T cell suspension volume can be up to 5 L or even more after the expansion step [106]. Thus, cell concentrating is necessary and conducted during the formulation/filling step to reduce the volume either for infusion or storage (hyperthermia storage or cryopreservation). The conventional concentration method requires multiple centrifugation steps, often leads to cell loss and clumping, and requires extensive labor [107]. Furthermore, the process of transferring and pooling centrifuged cell suspensions heightens the risk of contamination. Lastly, the centrifugal force has potential to damage the cell membrane, organelles

and DNS for various cell types [108, 109]. Consequently, there is an unmet need for innovative methods and devices designed for the large-scale concentration of cell suspensions.

In the cryopreservation and ATMP manufacturing process, CPA addition/removal and cell concentrating are usually conducted by different instruments. Generally, an effective cell processing setup should encompass sterile processing and automated procedures to reduce human workload. Additionally, it should incorporate protocols optimized specifically for each cell type. In this study, we have developed a novel 3-in-1 multifunctional cell processing system. This advanced system integrates the functionalities of CPA addition, CPA removal, and cell concentrating, all within a closed and sterile environment. In CPA removal, the system employs the dilution-filtration method and augmented the performance by a novel hypertonic dilution option, which reduces the cell volume excursion and osmotic injury. Furthermore, we also proposed an algorithm to generate optimal cell type-specific CPA removal protocols. To validate the system's performance, we conducted comprehensive experiments focusing on cell concentrating and cell-free CPA removal. These experiments yielded valuable insights and demonstrated the effectiveness of our approach.

2.2 Material and methods

2.2.1 Design and setup

The experimental setup, illustrated in Figure 2-1, comprises several key components to facilitate the CPA removal process. The system includes three peristaltic pumps, three 3-way T-pattern valves, an oscillating shaker, a hemodialyzer (F6HSF), and interconnected tubing. The peristaltic pumps serve as driving forces, propelling the liquid along the tubing to create circulation. The dialyzer membrane, designed with pore sizes much smaller than human cells,

effectively retains the cells within the cell loop, preventing their removal through ultrafiltration. Notably, Valve 1 can be switched between a high osmolality solution (hypertonic dilution solution) and an isotonic dilution solution to accommodate different dilution modes. Valve 2 and Valve 3 play crucial roles in recovering the residual cell solution left in the cell loop following the CPA removal and cell concentrating processes. Lastly, diluted cell suspension can be concentrated by following the loop by switching Valve 3. This multifaceted setup ensures the efficient and controlled execution of the CPA removal and cell concentrating procedures. CPA addition can be completed by turning pump 1 on and keeping pumps 2 and 3 off. By adjusting the speed of the pump 1, different CPA addition rates can be achieved, which allows the user to control the cell volume change rate. Concentration mode can be co-operated with CPA addition mode when cell suspension volume needs to be maintained or reduced. Table 2-1 demonstrates the control setting for different modes.

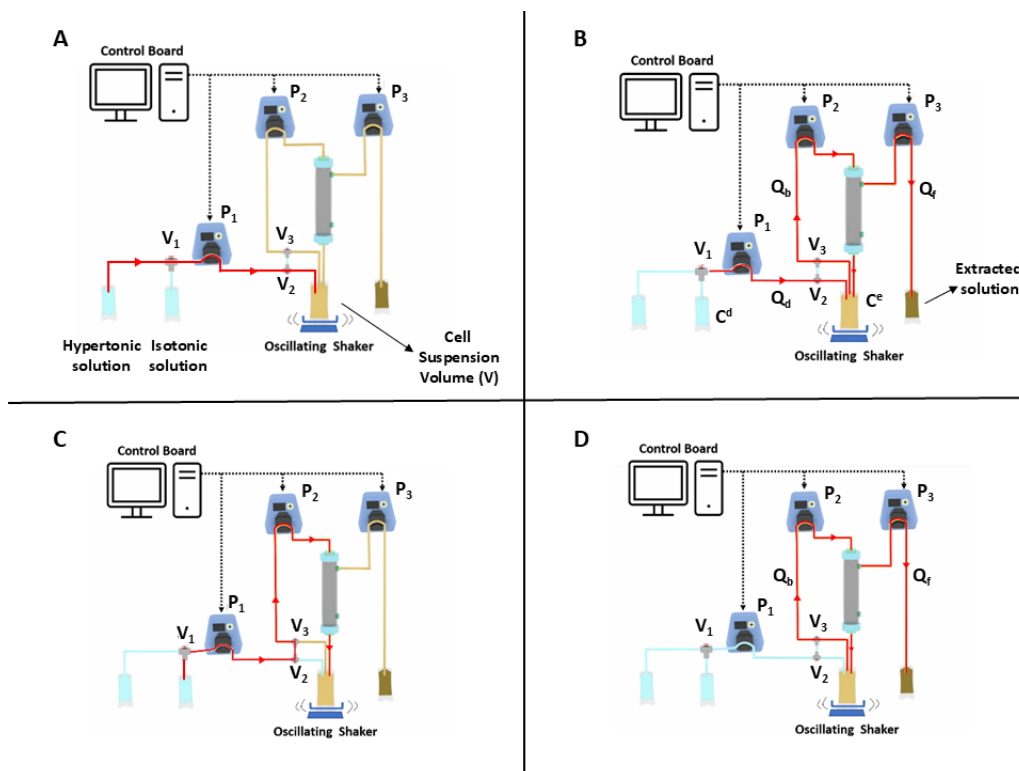


Figure 2-1. Diagram of the multifunctional cell processing system with three function flow diagrams. (A) CPA addition mode, (B) CPA removal mode, (C) Cell retrieval mode, and (D) Cell concentrating mode. Note: P represents

the pump and V is three-way valve, Q_d is dilution volume flow rate, Q_b is the blood side volume flow rate Q_f is ultrafiltration volume flow rate, C^d is the dilution osmolarity, C^e is the extracellular environment osmolarity.

TABLE 2-1. CONTROL SETTINGS FOR DIFFERENT WORKING MODES

Mode	P1	P2	P3	V1	V2	V3	Oscillating shaker
CPA addition	On	Off	Off	→	→	---	On
CPA removal	On	On	On	*	→	←	On
Cell retrieval	On	On	Off	↗	↖	↙	Off
Cell concentrating	Off	On	On	---	---	←	On

P represents the pump and V is three-way valve; arrow presents the flow direction in the three way valve. → shows flow direction is left to right, ←(right to left), ↗(bottom to right), ↖(left to top), and ↙(bottom to left); * represents the valve direction depends on isotonic dilution or hypertonic dilution; --- represents the valve is not applicable in current cycle.

2.2.2 Numerical simulation in selection of flow rate, dilution solution, and processing time

The assumptions have been made that: (1) Cell size (volume and surface area) and inactive volume V_b are assumed as identical with using previous measurement average from microfluidic device [93]; (2) Cell membrane permeability of water and CPA keep constant; (3) The dilution solution and cell solution will mix thoroughly and immediately when the dilution solution is pumped into the cell bag with an oscillating shaker, thus the osmolality within the cell loop (blood circuit in dialysis) is considered uniform; (4) During the CPA removal process, the extracellular osmolality was studied and used to set up a transient environment for cells; (5) Facing changing extracellular osmolality, cell volume changes were only dependent on water and CPA movements across the cell membrane; (6) The cell concentrating process was simulated based on the total

volume change between the initial and target of cell suspension; (7) CPAs cytotoxicity is not considered; (8) The simulation and figures are conducted with MATLAB (The Mathworks Inc.).

2.2.2.1 Extracellular osmolality

The cytocrit (α) can be calculated with the size, volume, and concentration of cells. For Jurkat cells, the diameter is 11.5 μm , and the volume is 796.33 μm^3 [110] with a concentration of 10^6 /mL. The cytocrit of the Jurkat cell can be calculated as 7.96×10^{-4} . By knowing the cytocrit (α) of experimental Jurkat cell suspension, the effects of intracellular water can be ignored during the extracellular osmolality calculation. Extracellular osmolality is dependent on both the volume dilution solution input and the ultrafiltration. However, the blood side volume flow rate, Q_b , doesn't affect the cell suspension volume. Thus, the extracellular osmolality is governed by the following equations:

$$\frac{dV}{dt} = Q_d - Q_f \quad (2-1)$$

$$\frac{d(C^e V)}{dt} = C^d Q_d - C^e Q_f \quad (2-2)$$

Where V is the total volume of the cell suspension, Q_d and Q_f are flow rates of dilution and ultrafiltration separately, C^e represents the osmolality of extracellular solution and C^d represents the osmolality of the dilution solution.

2.2.2.2 Cell volume change over time

The cell volume change is dominated by water and CPA transportation across the cell membrane during the CPA removal process, which can be calculated by two-parameter transport formalism [111] with obtained cell membrane properties and extracellular osmolality profiles. Equations 2-3, 2-4, and 2-5 were employed to simulate cell volume excursion.

$$\frac{dV_c(t)}{dt} = \frac{dV_s(t)}{dt} + L_p \cdot A \cdot (C^i - C^e) \cdot R \cdot T \quad (2-3)$$

where V_C and V_S are cell volume and intracellular CPA volume at time t , respectively. C^i , C^e are intra- and extracellular osmolarity, including CPA and saline, A is cell surface area, R is gas constant, and T is the temperature in kelvin. L_p is the cell membrane permeability coefficient to water.

The CPA flux can be calculated by the following two equations:

$$\frac{dM_s(t)}{dt} = P_s \cdot A \cdot (C_s^e - C_s^i) \quad (2-4)$$

$$M_s(t) = V_s(t)/v_s \quad (2-5)$$

where P_s is the cell membrane permeability coefficient to CPA, C_s^e , C_s^i are extra- and intracellular CPA contributed osmolarity, and $C_s^e = C^e - C[\text{saline}]$, $C_s^i(t) = M_s(t)/V_C(t)$. $M_s(t)$ is the mole of intracellular CPA at time t , v_s is the partial volume of CPA (mole/L).

2.2.2.3 Cell concentrating

The standard pore size of hollow fiber is around 200 nm, which is much smaller than most human cells [112]. In this work, the diameter of Jurkat cells is mainly from 10 μm to 16 μm . So that the cells do not pass across hollow fiber membrane but stay in the cell loop during the concentration process. As the fluid of cell suspension is removed by ultrafiltration, the total cell suspension volume gradually decreases, and the desired cell concentration will finally be reached. The volume flow rate of the dialyzer's blood side, Q_b , still doesn't change the cell suspension volume since it's circulating. The Eq 2-1 can be simplified by setting the Q_d equals to zero to

maximum the concentrating performance, which leads following equations to describe the cell suspension volume change rate during concentration mode:

$$\frac{dV}{dt} = -Q_f \quad (2-6)$$

$$n(t) = \frac{N}{V(t)} \quad (2-7)$$

where N is the total cell numbers in the cell suspension, and $n(t)$ (mL^{-1}) is the cell concentration at time t.

2.2.3 Cell-free CPA removal experiment

To compare the performances of CPA removal, three distinct groups were tested. In the following three tests, cell-free CPA solution (10% v/v DMSO in phosphate-buffered saline (PBS)) is used to test the performance of the CPAs removal by evaluating the osmolality at intervals of 1 min. Three batches of solution were prepared separately for group 1-3. The osmolality of three groups was measured with Vapor 5520 osmometer (Scimetrics, Inc.) Groups 1-3 compare the CPA removal performance by using the isotonic dilution-filtration method with varying flow rates (group 1-2) and the hypertonic dilution-filtration method (group 3). Detailed information can be found in Table 2-2:

Group 1 (Isotonic-Slow): 1X PBS was pumped into the loop from the tubing near the dialyzer's inlet with a speed of 20 mL/min. To keep the volume of the cell solution constant, the ultrafiltration rate was also set as 20 mL/min.

Group 2 (Isotonic-Fast): 1X PBS was pumped into the solution reservoir directly at a speed of 100 mL/min. The ultrafiltration and cell suspension volume flow rate was set as 100 mL/min as well to keep the cell solution volume constant.

Group 3 (Hypertonic-Isotonic-Fast): The flow rates of addition and ultrafiltration were the same as group 2. The additive solution was changing between hypertonic DMSO solution and isotonic saline solution. By using a multi-step dilution solution, the dramatic change of the osmolality can be reduced and potentially increase the cell survival rate.

TABLE 2-2. SETUP OF CELL-FREE CPA REMOVAL EXPERIMENTS

	Group 1	Group 2	Group 3
Pump 1 (mL/min)	20 mL/min	100 mL/min	100 mL/min
Pump 2 (mL/min)	20 mL/min	100 mL/min	100 mL/min
Pump 3 (mL/min)	20 mL/min	100 mL/min	100 mL/min
Total Volume (mL)	1110	1110	1113
Osmolality of Dilution Fluid (mOsm/kg)	292	292	1153 (0-60s) 549 (61-120s) 376 (121-180s) 292 (181s-)

2.2.4 Cell concentrating

2.2.4.1 Cell preparation

Human acute lymphoblastic leukemia cells (Jurkat cells) used in this work were purchased from ATCC (American Type Culture Collection, Manassas, VA) and cultured in a 37°C incubator with 5% carbon dioxide and proper humidity. 500 mL culture media consists of 440mL RPMI medium 1640 (Life Technologies), 50 mL fetal bovine serum (FBS), 5mL penicillin-streptomycin, and 5 mL L-glutamine. Cells with a density of $\sim 10^6/\text{mL}$ were used for experiments.

2.2.4.2 Cell concentrating experiments

The cell concentrating experiment was conducted using the 3-in-1 multifunctional cell processing system (Table 2-1 cell concentrating mode). The experiment follows the procedures below:

- (i) Dilute the pre-prepared 50 mL cell solution with a cell concentration of 4.4×10^5 cells/mL to 250 mL;
- (ii) Priming the whole system (40 mL priming volume) with PBS for 5 min to remove air bubbles, affecting concentration performance;
- (iii) Circulate the diluted cell solution into the cell processing system with cell circulation (P2) and ultrafiltration pump (P3) operating at 15 mL/min. Then, collect the solution same at the beginning and then every 2 minutes for cell counting;
- (iv) Once the cell solution bag closing to empty, remove the tube and turn off the ultrafiltration pump (P3). Then, retrieve 42 mL cell solution (by collecting the cell solution remaining in the tubing back to the cell bag);
- (v) As for the cell counting process, mix 10 μ L cell suspension with 10 μ L Trypan Blue solution (Sigma-Aldrich) and count with Countess II FL Automated Cell Counter (Invitrogen).

2.3 Results

2.3.1 Numerical results and experiment results for CPA removal

2.3.1.1 The removal performance of three approaches and validation of the CPA removal model

As seen in Figure 2A, extracellular osmolality profiles of three different CPA removal approaches are shown. The initial osmolality of the three groups are 1859, 1715, 1650 mOsm/kg, respectively. The variation between the three is attributed to differences in solution preparation. The experimental initial osmolality data was applied to the corresponding group in theoretical models. After 18-min experiments, the osmolality of group 1 (isotonic-slow) dropped to 1462 mOsm/kg while these of the other two groups were 570 and 591 mOsm/kg, respectively. Numerical simulation based on Equations (1), (2) has been conducted and plotted in Figure 2A. Therefore, the model was validated since it shows great consistency between experimental data and numerical

simulation results and will be further used in the investigation and optimization of the 3-in-1 cell processing system. Figure 2B demonstrates the extracellular osmolality environment change rate in group 2 and 3. As shown in Figure 2B, the group 3 contains 3 switch point, aligned with 3 times of switching dilution solution, and the extracellular osmolality changing rate of group 3 tends to close group 2 with time increases because the osmolality difference become smaller between two groups. Normalized residual CPA is used to determine the remaining intracellular CPA, which is ratio between the current and initial intracellular CPA concentration. Figure 2C shows the normalized residual CPA of different groups when initial osmolality is set at 1800 mOsm/kg. The curves of groups 2 (isotonic-fast) and 3 (hypertonic-isotonic) are precisely the same since every parameter regarding the CPA is the same. Also, since the volume flow rate of the dilution in group 2 and 3 is sufficient enough to drive the CPA out, the normalized residual intracellular CPA is very close, but the cell volume changes are different as discussed in section 3.1.2.

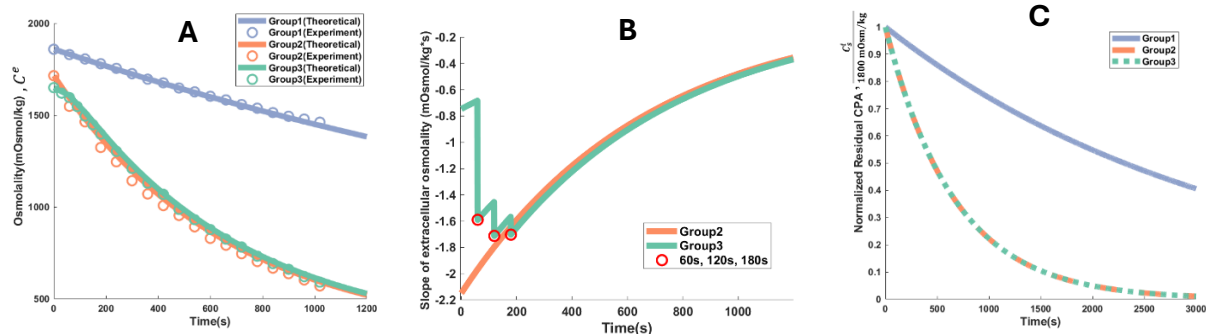


Figure 2-2. Simulation of CPA removal process. (A) Simulation and experiment results of cell-free CPA removal experiments. Group 1 represents the Isotonic-Slow; Group 2 is the Isotonic-Fast and Group 3 is the Hypertonic-Isotonic-Fast. Detailed operation described at Table 2-2. (B) The osmolality change rate of extracellular environment for group 2 and 3. (C) Normalized residual CPA ($\frac{C_s^i}{1800 \text{ mOsm/kg}}$), with Group 1, 2 and 3 removal approaches.

2.3.1.2 Cell volume change of different cell types and different CPA removal approaches

The numerical extracellular osmolality profiles had been validated in the previous subsection and then further used to calculate cell volume change during the CPA removal. Jurkat cells are an immortalized line of human T lymphocytes often used in laboratory research. Meanwhile, since

cryopreservation of the immune cells enhances the development of advancing cellular therapies, which is crucial in both systemic and local immune responses to improve therapeutic outcomes in cancer, infectious diseases, and reproductive health applications, vaginal T cells and vaginal macrophages were also selected in this study. Thus, three types of human cells, Jurkat cells, vaginal T cells, and vaginal macrophages, were investigated for their cell volume reactions with different CPA removal approaches discussed in section 2.2.3. Related cell properties and membrane permeabilities used during simulation can be found in Table 2-3. The initial condition was set as 10% DMSO (1800 mOsm/kg). The hypertonic saline solution of group 3 had been modified to 500 mOsm/kg for the first half minute. And then, the isotonic saline was used as a dilution solution starting from the 31st second.

As shown in Figures 2-3 (A-C), normalized cell volumes, cell volume at time t divided by the initial cell volume, were calculated for each removal approach. For Jurkat cell, group 1 has the smallest maximal cell volume, 1.012, during the CPA removal process. Group 2 and 3 have relatively similar peak values, which are 1.048 and 1.035, respectively. For vaginal T cells, the maximal cell volumes for groups 2 and 3 are 1.022 and 1.009, respectively, while group 1 is 1.005. Vaginal macrophages got 1.004 for group 1 and 1.017 for group 2. The maximal cell volume of group 3 is 1.003, which is even smaller than group 1.

TABLE 2-3. CELL PROPERTIES AND MEMBRANE PERMEABILITIES WITH 10% M/V DMSO

Cell types	Diameter (μm)	V_b	L_p ($\mu\text{m}/\text{min}/\text{atm}$)	P_s ($\mu\text{m}/\text{min}$)
Jurkat Cells [110, 113]	11.5	49.6%	0.14	0.34
Vaginal T Cells [93]	8.43	51.6%	0.089	0.47
Vaginal macrophages [93]	9.62	45.7%	0.234	0.99

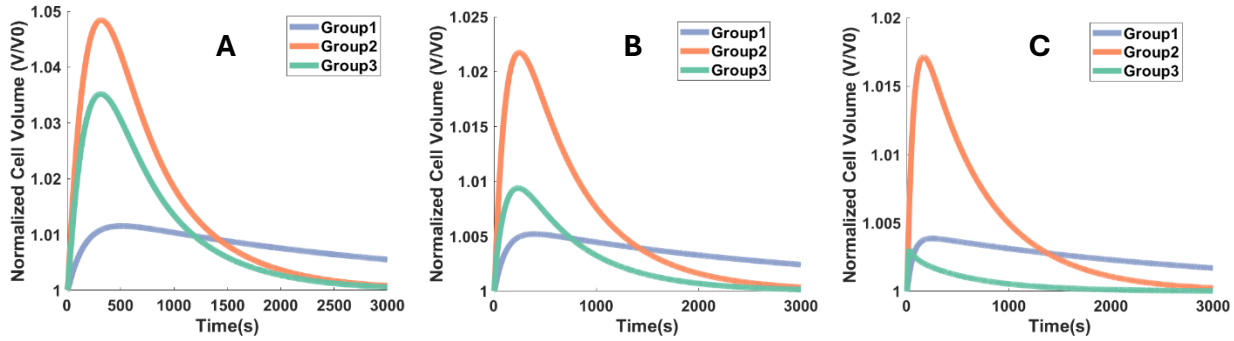


Figure 2-3. Cell volume change with different CPA removal methods. (A) Jurkat cells volume change with CPA removal approaches under group 1, 2 and 3; (B) Human vaginal T cells volume change different CPA removal approaches under group 1, 2 and 3; (C) Human vaginal macrophages volume change with different CPA removal approaches under group 1, 2 and 3.

2.3.1.3 Selection of the fastest dilution rate with the isotonic dilution method

Based on the simulation of volume changes using different approaches discussed in the preceding subsection, it was observed that the group 1 (isotonic-slow) method requires an excessive amount of time, exceeding one hour, for complete CPA removal. Consequently, group 1 was not chosen for further consideration and simulation. In contrast, group 2 (isotonic-fast) and group 3 (hypertonic-isotonic) were respectively renamed as the isotonic and hypertonic methods, based on the specific dilution solutions employed. In order to provide a clearer demonstration of cell volume changes, the initial osmolality of the cell suspension was increased to 4000 mOsmol/kg. It is worth noting that CPA solutions with such high osmolality, or even higher, are referred to as vitrification solutions, which are commonly utilized in cryopreservation to prevent ice formation during the cooling process [22].

Once the isotonic method is chosen, as illustrated in Figure 2-4A, the rates of CPA removal are directly influenced by the flow rates of dilution and ultrafiltration, which are maintained the same throughout the process to ensure a constant volume. Faster dilution and ultrafiltration result in quicker CPA removal. However, it is important to note that increasing the dilution rate can lead to more pronounced cell swelling, as depicted in Figure 2-4B. As seen in Figure 2-4C, this creates

a trade-off situation between the speed of CPA removal and the extent of cell excursion. The multifunctional cell processing system offers a customizable standard for cell excursion. For instance, when 5% expansion is selected as the maximum tolerance for Jurkat cells, Figure 2-4C indicates that the maximal flow rate under this constraint is 39 mL/min. This editable standard enables the optimization of CPA removal while carefully controlling cell excursion to ensure the preservation of cell viability.

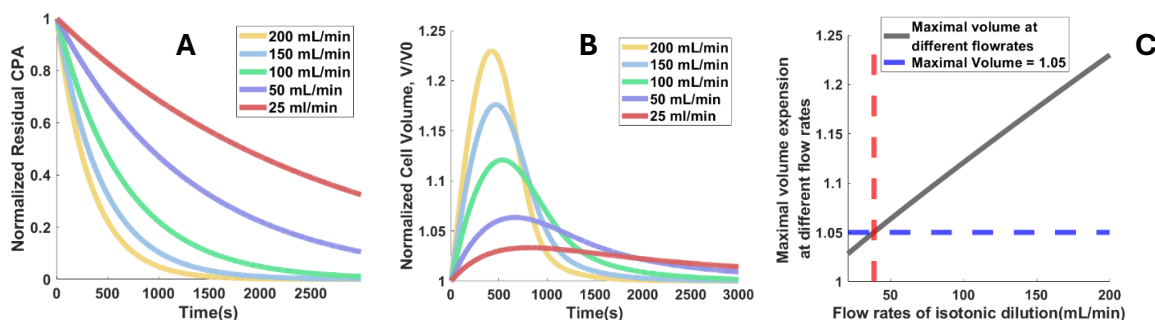


Figure 2-4. Volume flow rate effect on CPA removal. (A) Normalized residual intercellular CPA with varying dilution rates on isotonic mode (1X PBS); (B) Normalized cell volume change with varying dilution rates for Jurkat cell in isotonic mode; (C) Selection curve for isotonic mode when cell volume expansion tolerance is 5% to the normal cell volume.

2.3.1.4 Selection of hypertonic dilution methods to obtain minimal volume change

Once the hypertonic mode is selected, the cell suspension will be diluted by hypertonic solution in the first half minute and isotonic solution in the remaining time. The osmolality of hypertonic saline solution has a considerable impact on the cell volume change during CPA removal. The cell volume changes of Jurkat cells, vaginal T cells, and vaginal macrophages with different hypertonic saline solutions in the first half minute is shown in Figure 2-5A-C with enlarged first 50s. According to the simulation results, the higher the osmolality of the hypertonic saline solution, the lower peaks of cell volume curves would be obtained. Once the osmolality is more significant than a particular value related to the cell membrane properties, cells would suffer a shrinkage after a small peak at the very beginning. With a higher osmolality, the curve will obtain

a deeper valley. This phenomenon could help us obtain a theoretical optimal CPA removal protocol in hypertonic mode. The cell volume change will be accumulated and compared. No matter what initial conditions it has, the “selection curve” shown in Figure 2-5D will give us a theoretical optimal hypertonic solution to achieve minimal cell excursion. Figure 2-5D shows Jurkat cells, vaginal T cells, and vaginal macrophages as examples. From Figure 2-5D and its cryobiological properties at Table 2-2, the cell contains larger P_s can be diluted with lower osmolarity solution, which means larger osmolarity gradient between extracellular and intracellular environment.

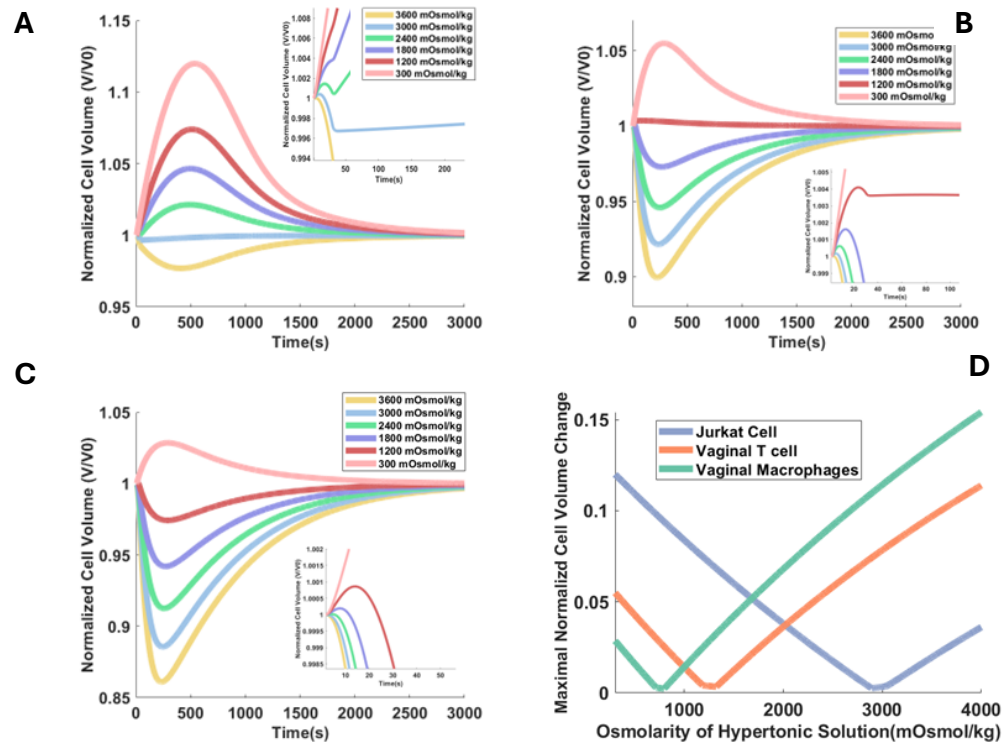


Figure 2-5. Various CPA removal simulation with initial hypertonic solution. (A–C) Normalized cell volume changes with the varying hypertonic solution (changing osmolarity) in a first-half minute on the hypertonic mode and then switch to isotonic mode. (A) Jurkat cells; (B) Vaginal T cells; (C) Vaginal macrophages; (D) Selection curve for hypertonic mode to determine the concentration needed for first-half minute for three cell types, which is crucial to minimize the cell volume change.

2.3.2 Numerical results and experiment results for cell concentrating

The concentration experiment results are depicted in Figure 2-6. The theoretical curve was plotted using Equations (6) and (7). Cell concentrations were measured three times at 2-minute intervals. The final recovery rate of Jurkat cells was determined by the ratio between the final and initial live cell number, which was 86.0% in cell concentrating experiments. These findings provide valuable insights into the effectiveness of the cell concentrating process and demonstrate a promising recovery rate for Jurkat cells.

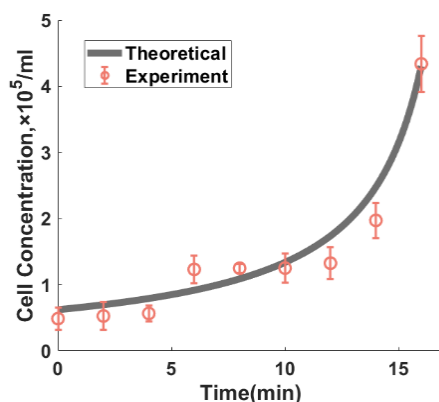


Figure 2-6. Cell concentrating mode of the multifunctional cell processing system between experiment and theoretical results using Jurkat cell as representation.

2.4 Discussion

The 3-in-1 multifunctional cell processing system is necessary to improve the CPA removal and further improve the cell preparation process. It highly decreases the processing time and osmotic injury during the CPA removal process. Compared with the traditional dilution-centrifuge method, the 3-in-1 multifunctional cell processing system can reduce cell clumping and loss, stem cell activation, and contamination risk. Another huge advantage of the system is that it is capable of CPA addition, removal, and cell concentrating automatically. Pumps and valves in this closed system can be controlled by PC or other processors without manual operation required during the

centrifugation process, which will introduce personal error and uncertainty. Cell type-dependent algorithms are built to optimize the scenarios of CPA removal. A cell-free experiment first validated the simulation model. The results showed that the assumptions are reasonable, and theoretical results can be used as a reliable reference for further investigation.

According to the cell volume results based on the validated numerical model, cell volume excursion is mainly determined by cell properties, including L_p , P_s , diameter, and inactive volume. Comparing the results with the same dilution protocol shown in Figure 3, and Table 2, three types of cell perform different behaviors during the CPA removal procedure due to the varying osmotic properties listed above. The larger L_p leads to a faster water transport rate across the cell membrane, which brings the cell excursion peak at earlier time. The smaller P_s value results in slower rate of intercellular CPA pass out the cell membrane showing a larger normalized cell volume. Generally, traditional low-speed isotonic dilution (group 1) would cause very mild osmotic injury to cells. However, it takes too much time for the CPA removal process, and the cell would stay in a high-CPA solution for a long time, which might induce cell damage caused by the toxicity of CPA. High-speed isotonic dilution and hypertonic dilution (groups 2 and 3) have similar CPA removal performance. Hypertonic dilution can further reduce osmotic injury by balancing water influx and CPA outflux of cells. Based on the results, as for CPA removal starting from middle-level osmolality, such as 1800 mOsmol/kg for 10% DMSO, the cell excursion is less than 5%, no matter which dilution mode is selected. The isotonic mode could be a potential option if mild cell excursion can be tolerated since the isotonic mode is relatively simpler to operate. But if the vitrification solution or other high-CPA solution is the operation sample, the hypertonic mode will be the desired selection to reduce severe cell swelling.

In other words, with the given cell properties, the specific CPA removal protocol or treatment can be generated by the algorithm derived from mass transfer equations. Based on this theory, isotonic and hypertonic modes have been further improved. As shown in Figures 4 to 5, the isotonic mode pursues simpler and faster removal under a given tolerance, while the hypertonic mode looks for minimal osmotic injury.

Cell concentrating mode can be operated individually or included as part of post-cryopreservation procedures, depending on needs. In-vitro experiments have been conducted, and the results are auspicious.

Besides developing the multifunctional cell processing system and algorithms, the exciting relation between cell cryobiological properties, including L_p , P_s , diameter, and inactive volume, was also noticed. By employing our pre-development microfluidic device [113], we are able to determine the cryobiological properties at different temperatures, which creates an opportunity for development of CPA removal protocols with temperature change, especially when samples pass the melting point, for various cell types as a pre-run test. Moreover, Yadegari et al. proposed a model to simulate the osmotic injury during the cooling and rewarming processes, which is also greatly beneficial for our future work when applying our cell process systems where there are temperature changes or fluctuations [114]. In a specific combination of these parameters, the cell can balance its water influx and CPA outflux during the CPA removal process. It could explain why some cell types are more accessible to be successfully cryopreserved while others are not. We will keep investigating this phenomenon in our future research. In addition, although two parameter model was used in this study, the 3-in-1 cell processing system is also capable to adopting the newly proposed hypertonic volumetric equilibration model by using the Boyle van't Hoff (BvH) considering more cellular component like, cytoskeleton and actin cortex,

mechanosensitive channels, and ion pumps [115, 116]. For other cells not following the two-parameter model, such as mesenchymal stem cells [117], the trans-membrane permeation of solutes during swelling phase should be considered. Moreover, since the cytotoxicity of CPAs such as DMSO [118, 119], the temperature control of the 3-in-1 cell processing system is desired to better improve the cell viability during all types of cell processing. Overall, the 3-in-1 cell processing system holds a great potential for adopting various cell types with different numerical models and provide guidance to operators.

2.5 Conclusion

A 3-in-1 multifunctional cell processing system, along with a computer-guided CPA removal protocol, has been developed to fulfill CPA addition, removal, and cell concentrating functions. By integrating an optional hypertonic dilution method and employing an algorithm that takes into account cell type-specific properties as parameters, the system generates an optimal approach for removing CPAs to minimize cell swelling. In comparison to the conventional dilution-centrifugation method, this innovative system and method offer a sterile, automatic alternative with reduced risk of osmotic and mechanical injury for various cell types. These advancements, both CPA addition/removal and cell concentrating, present promising options for academic and clinical research in the field.

Chapter 3. DEVELOPMENT OF NEW VITRIFICATION SOLUTION BY INTRODUCING POLYVINYLPIRROLIDONE

3.1 Introduction

Vitrification has proven to be a promising alternative cooling method, especially for tissues and organs, which contain multiple cell types [27, 28]. However, a common method to lower the required CCR is increasing the concentration of the permeating CPAs, such as VS55, DP6 and 7.05M DMSO [120]. However, DMSO has inherent toxicity that can cause apoptosis, development disorder, DNA/RNA disruption, and impaired enzyme functions [17, 121] and increasing concentration of DMSO increase those issues and also increase osmotic injury. Osmotic injury describes the free water of cells extract beyond the shrinkage limitation, which relies on the osmolarity gradient between cells and CPAs solution environment [19]. Thus, there is a need to decrease the concentration of the single group of CPAs, for example, Fahy development the M22 [77] to reduce the both of the toxicity and osmotic injury, but still being able to achieve vitrification. In addition, ice-blocker and anti-freezing protein are also popular but at high cost [122-124].

Although significant innovations have been developed in vitrification solution studies, these solutions still need further refinement to achieve uniform heating distribution and minimize cellular damage during rewarming. The vitrification solution design should focus on the following parameters: 1) vitrification tendency, 2) osmolarity, 3) viscosity, and the potential benefits for EM rewarming.

Polyvinylpyrrolidone has been employed in multifarious fields such as paper, fibers and textiles, ceramics, and pharmaceuticals due to its superior properties [125]. PVP is a water-soluble polymer made from the monomer N-vinylpyrrolidone. It is an inert, non-toxic, temperature-

resistant, pH-stable, biocompatible, biodegradable polymer available in various molecular weights ranging from 10,000 to 360,000 MW. In addition, PVP has glass transition temperature ranging between 370 – 430 K [126, 127], which can be used to increase the glass transition temperature of the cocktailed CPAs. Fahy proposed the use of 6% polyvinylpyrrolidone as non-permeating CPA in 1984 [27]. Smillie utilized the 5% PVP (10kDa and 40kDa MW) in preserving the human platelets [128]. Later, El-Shewy applied 10% PVP (40kDa MW) to preserve the islet cell [129]. Ren verified the vitrification ability of DPVP and identify the unique inverted U-shape dielectric properties to overcome the thermal runaway effect in the dielectric heating.

The chapter illustrates the new vitrification solution design with different concentration and molecular weight of PVP. The higher the molecular weight, the lower the solubility in DMSO/water solvent. Therefore, we studied the various molecular weight including, 10 kDa, 58 kDa, and 360 kDa and determined the mechanical, thermal and electric properties are determined. Further, the solution was experimented to test the vitrification ability at 20 mL volume to bring the future potential of tissue and animal organ vitrification.

3.2 Material preparation

DMSO is the permeating CPA used. The CPA includes DMSO, PBS and PVP. Detailed composition is shown in Table 3-1. The PVP was first weighted and then added designated mass of DMSO. Finally, PBS was added to the desired final mass. The 360 kDa is hard to dissolve into the DMSO/PBS solution and the maximum concentration we applied is 8%, which requires overnight stirring. The 10 kDa PVP performs a good solubility due to the smaller MW. The 10 kDa and 58 kDa PVP were purchased from Sigma-Aldrich (US). The 360 kDa were purchased from two vendors, Sigma-Aldrich (US) and Thermo Fisher Scientific (US).

TABLE 3-1. PROPOSED VITRIFICATION SOLUTION COMPOSITION

Name	DMSO (m/m%)	PVP (m/m%)	PBS (m/m%)
10D37.5PVP	10	37.5% (10 kDa)	52.5
10D50PVP	40	50% (10 kDa, sigma)	50
20D30PVP	20	30% (10 kDa)	50
30D20PVP	30	20% (10 kDa)	50
30D20PVP	30	20% (58 kDa)	50
40D6PVP	40	6% (360 kDa, Thermo Fisher Scientific)	54
40D6PVP	40	6% (360 kDa, Sigma-Aldrich)	54

3.3 Determination of the thermal properties of CPA solutions

3.3.1 Heat Capacity

Heat capacity is a temperature-dependent property. As shown in Figure 3-1, differential scanning calorimetry (Discovery DSC 2500, TA Instruments, DE, USA) was employed to precisely measure the difference in heat flow between CPA solutions and reference solutions over a temperature range from -85 °C to 20 °C. The isothermal step-scan method was implemented to minimize experimental error. Heat capacity was calculated using the following equation:

$$C_P = \frac{\Delta Q_{DSC}}{m\Delta T} \quad (3-1)$$

where ΔQ_{DSC} is the heat flow through the sample solutions captured by DSC. m stands for the mass of the sample. T is the temperature.



Specifications

Baseline Flatness (-50 to 300°C)	≤10 μW
Baseline Repeatability (-50 to 300°C)	<20 μW
Temperature Range (as installed)	-90 to 725°C with RCS 90 cooler
Temperature Accuracy	±0.05°C
Temperature Precision	±0.008°C
Enthalpy Precision	±0.08%

Figure 3-1. DSC equipment and its specification. TA Discovery DSC 2500 with auto loading system for thermal property measurement and its specification

Due to the limitation of the DSC temperature range shown in Figure 3-1 (right). Only 10D50PVP was tested using the TA2500. Sample solutions were repeated three times.

3.3.2 Glass transition temperature

The glass transition temperature (T_g) of 10D50PVP can be also studied by using the DSC. The T_g of the rest solutions are predicted using the fox theory [130]. Nyamwea et al. stated a simplified Fox equation as [131]:

$$\frac{1}{T_g} = \frac{w_1}{T_{g1}} + \frac{w_2}{T_{g2}} + \frac{w_3}{T_{g3}} \quad (3-2)$$

Where the T_g is the CPA cocktail glass transition temperature and the T_{g1}, T_{g2}, T_{g3} represents the components glass transition temperature, w_1, w_2, w_3 are the weight fraction of the components.

Table 3-2 shows the T_g of the CPA composition proposed in Table 3-1, which is a reference when using the Fox equation in predicting the T_g of vitrification solution.

TABLE 3-2. GLASS TRANSITION TEMPERATURE OF CPA COMPONENTS

Name	T_g (K)	Reference
DPVP (10kDa)	370	
DPVP (58kDa)	415	[127]
DPVP (360kDa)	423	
Water	136	[132]
DMSO	149.5	[133]

3.3.3 Results and Discussion

Figure 3-2 shows the heat capacity average and standard derivation from 3 duplicated experiments. The sample heat capacity increases with increasing temperature and there is no crystal-liquid phase change peak while there is a step showing the passing of the glass transition temperature.

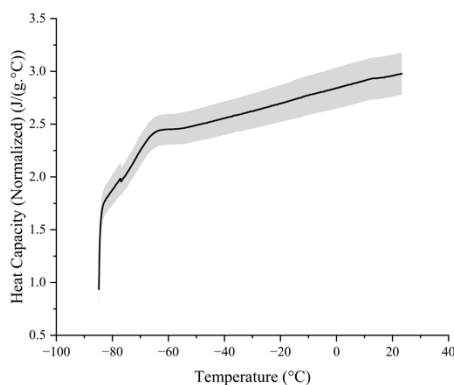
**Figure 3-2. Heat capacity of 10D50PVP (10kDa)**

Figure 3-3 presents an example of a heat flow diagram and its T_g capture of 10D50PVP by using the TRIOS software (TA Instrument, DE, USA). Three DSC experiments yielded measured T_g of -71.16 °C, -68.91 °C, and -71.42 °C, resulting in an average T_g of -70.50 ± 1.38 °C. The

predicted T_g , calculated using the Fox equation, is $-71.44\text{ }^{\circ}\text{C}$, corresponding to a prediction error of approximately 1.3%.

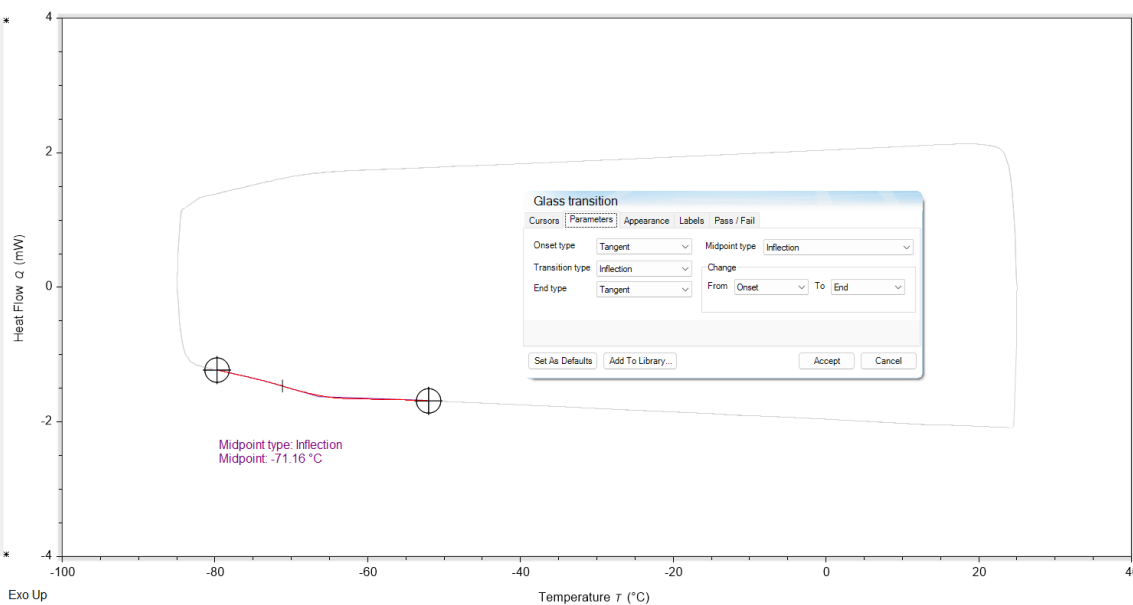


Figure 3-3. Heat flow diagram and highlighted T_g of 10D50PVP

Table 3-3 shows the predicted T_g by using the Equation 3-1 and Table 3-2. The results show that the increasing PVP concentration reduce the T_g making the solution easier to be vitrified. In addition, although the higher MW leads to higher T_g when same concentration is applied, the difference is small. For example, the difference of T_g of 30D20PVP between 10 kDa and 58kDa is $1.52\text{ }^{\circ}\text{C}$, but the viscosity is significantly increased and discussed in Chapter 3.4. Therefore, the lower MW PVP is a better option in designing the vitrification solution since its better solubility and less viscosity.

TABLE 3-3. PREDICTED T_g OF VITRIFICATION SOLUTION

Name	Predicted T_g (K)	Predicted T_g (°C)
10D37.5PVP (10 kDa)	180.42	-92.58
20D30PVP (10 kDa)	171.67	-101.33
30D20PVP (10 kDa)	160.68	-112.32
30D20PVP (58 kDa)	162.20	-110.80
40D6PVP (360 kDa)	147.32	-125.68

3.4 Determination of Viscosity of CPA solutions

3.4.1 Viscosity measurement

Viscosity plays a critical role in vitrification including the prevention of ice nucleation, reducing the required CCR, and recrystallization inhibition [134-136]. Higher viscosity results in greater kinetic barriers preventing the water molecular rearrangement of water to form ice, but hard to transfer with pipette. Viscosity was tested with Discovery HR30 Rheometer (TA instruments, DE, USA) at the University of Washington (Tacoma), shown as Figure 3-4.



Figure 3-4. Discovery HR30 rheometer during operation

The procedure and setting parameter of the is shown as Figure 3-5. The cooling rate was set as 10 °C/min. The testing sample volume is 5 uL and the testing range is -100 °C to 20 °C.

1: Flow Sweep

Environmental Control

Temperature °C ☐ Inherit Set Point

Soak Time s ☒ Wait For Temperature

Test Parameters

Logarithmic sweep

Shear rate 1/s to 1/s

Points per decade

☐ Steady state sensing

Equilibration time s

Averaging time s

☐ Scaled time average

☒ Controlled Rate Advanced
☒ Data acquisition
☒ Step termination

Figure 3-5. Rheometer procedure and setting parameter

3.4.2 Results and Discussion

Figure 3-6 shows the viscosity results ranging from -100 °C to 30 °C of the 30D20PVP (10 kDa), 30D30PVP (10 kDa), 30D20PVP (58 kDa), 40D6PVP (360 kDa) and VS55 [134]. The viscosity increases with increasing PVP concentration. When DMSO and PVP concentration remain the same, the viscosity is similar between 10 kDa and 58 kDa. However, when the PVP MW increases to 360 kDa, the viscosity significantly increases during board temperature range. Since viscosity limits the ice growth during the cooling process and it is a function to temperature, those solutions have higher viscosity between the melting temperature and T_g is desired. From the Figure 3-6, the viscosity presents similar value when temperature around -100 °C to -80 °C. Thus, a better handling solution with less viscosity at the 0 °C or 20 °C is selected during our CPA design protocol.

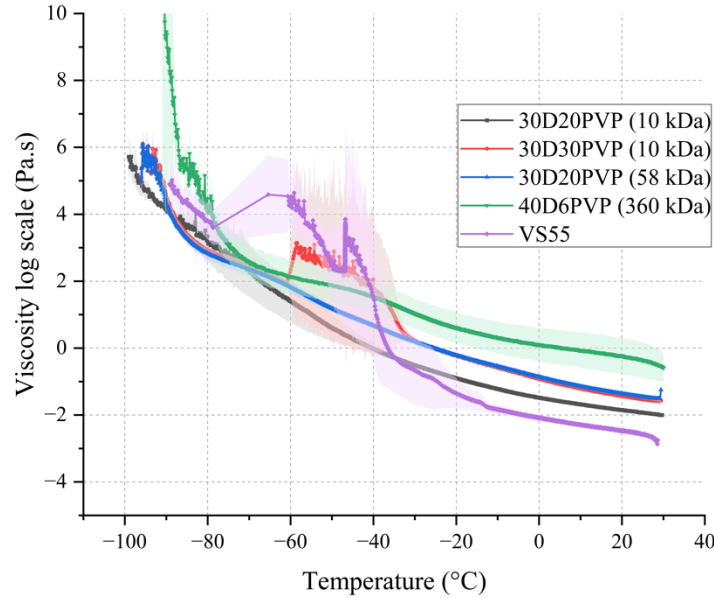


Figure 3-6. Vitrification solution viscosity with different PVP MW and concentration

3.5 Determination of osmolality of CPA solutions

3.5.1 Osmolality calculation

Osmolarity describes the moles of osmotically active particles per liter of solution (mOsm/L) or per kg of solvent (mOsm/kg). Osmotic injury is one of the biggest problems of the vitrification solution as it requires high concentration of the CPA combinations. However, the large MW polymer may be an alternative option in achieving vitrification, meantime, large MW of PVP results in low mole mass in the solution even high concentration is used. Here we calculated and tested osmolality of CPA solution described in Table 3-1. The fundamental equation of osmolality is:

$$Osmolarity \left(\frac{mOsm}{L} \right) = Molarity (M) \times Van't Hoff Factor \times 1000 \quad (3-3)$$

Where the Molarity is the mole mass of the solute and the Van't Hoff Factor is defined as

$$i = \frac{\text{apparent number of particles in solution}}{\text{number of moles of solute dissolved}}, \text{ which material type dependent [137].}$$

3.5.2 Osmolality measurement

The osmolality measurement has been conducted in K-7400S Semi-Micro Osmometer (KNAUER, Germany) shown in Figure 3-7.



Figure 3-7. Setup of Osmometer

The osmometer was first calibrated with the standard solution and the measurement range is 0 – 2000 mOsmol/kg with $SD \leq 4$ mOsmol/kg. As the vitrification solution is typically beyond the range of the osmometer, dilution is needed. The operation follows and each sample was tested three times ($n = 3$):

1. Numerical calculation to determine if the solution needs to be diluted
2. Dilute the solution to fit the equipment range (10X dilution)
3. Draw 150 μ L solution to the sample holder and attached the sample holder with the measurement tip

4. Start the measurement and wait for 80s

3.5.3 Results and discussion

Table 3-4 shows the calculated (assume PBS osmolality is 300 mOsmol/kg) and measured osmolality from the Table 3-1 proposed vitrification solution. The increasing DMSO significantly increases the osmolality as its MW is 78.13 g/mol. However, PVP MW is ranging from 10 kDa to 360 kDa, which makes only small osmolality increase when using lower MW PVP from calculation. The measured results align with the calculated results and the variation may result from dilution and molecular interaction. The commonly used CPA, 10% DMSO, has osmolality is around 1400 mOsmol/kg and new proposed CPA, 10D37.5PVP (10 kDa) shows similar osmolality, but bring the potential in large volume cell vitrification.

TABLE 3-4. PREDICTED T_g OF VITRIFICATION SOLUTION

Name	Calculated Osmolality (mOsmol/kg)	Measured Osmolality (mOsmol/kg)	Relative Error (%)
10D37.5PVP (10 kDa)	1475	1570	6.05
20D30PVP (10 kDa)	2470	2556.67 ± 4.71	3.39
30D20PVP (10 kDa)	4010	4383.33 ± 49.89	8.52
30D20PVP (58 kDa)	3993	3436.67 ± 95.33	16.18
40D6PVP (360 kDa)	5282	5366.67 ± 4.71	1.57

3.6 Determination of Dielectric Properties of CPA solutions

Multiple methods can be used to measure dielectric properties, including the coaxial probe, transmission line, free space, resonant cavity, and parallel plate. The coaxial cable, transition line,

and free space methods can be tested on broadband frequencies. The coaxial cable method is convenient because it requires minimal sample preparation and less sample volume.

3.6.1 Coaxial probe measurement for CPAs in broadband frequency (200 MHz – 1.5 GHz) at room temperature

Dielectric properties of common CPAs are measured using a coaxial probe (Keysight N1501A) for both pure and binary solutions to understand how the dielectric properties behavior for different CPAs and concentrations, which is significantly influenced by the hydron bond effect between solute and solvent. Figure 3-8 shows the measurement coverage of the solution.

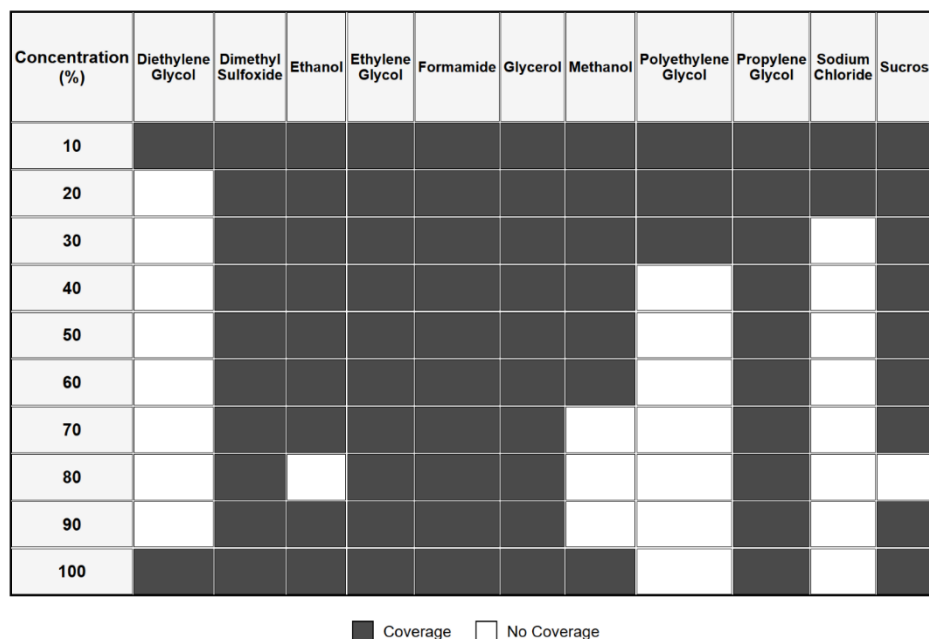


Figure 3-8. Measured Solution heatmap using coaxial cable method

Figure 3-9 demonstrates the device's setup. Calibration was performed using the standard open-short-load procedure, employing air as the open standard, the probe's built-in shorting block as the short standard, and deionized (DI) water as the load (a known dielectric standard). Following calibration, the coaxial probe was vertically inserted into the liquid cryoprotectant sample, with its

tip positioned at the vertical center of the container to prevent edge effects and ensure uniform exposure of the electromagnetic field. The measurement relies on the reflection coefficient (S11) recorded by a vector network analyzer (Agilent E5061B). S11 is the negative of return loss, which describes the amount of power that is reflected from the test material.

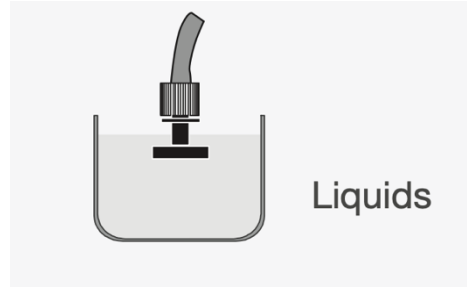


Figure 3-9. Setup of coaxial cable measurement of dielectric properties

3.6.2 Cavity perturbation method for CPAs at subzero temperature

The cavity perturbation method works for single-frequency dielectric measurement, and it can test samples in various temperatures and phases. It relies on the cavity perturbation theory and is governed by the equations below.

$$\varepsilon'_r - 1 = \frac{(f_0 - f_s) V_c}{2f_s V_s} \quad (3-4)$$

$$\varepsilon''_r = \left(\frac{1}{Q_s} - \frac{1}{Q_0} \right) \frac{V_c}{4V_s} \quad (3-5)$$

Where the ε'_r is the relative dielectric constant and the ε''_r is the relative dielectric loss. f_0 is the resonant frequency of the empty cavity and f_s is the resonant frequency after inserting the test sample. V_c is the cavity volume and V_s is the sample volume. Q_s is the quality factor of the loaded cavity and the Q_0 is the quality factor of the empty cavity.

To ensure accuracy, the relative resonance frequency change between the empty cavity and loaded cavity should follow $(f_0 - f_s) / f_s \leq 10^{-3}$. The test was conducted using a 434 MHz and 915 MHz cavity with a vector network analyzer (Agilent E5061B).

To standardize the measured data, a calibration has been conducted by using equations below to determine the C_1 and C_2 factors [138].

$$\Delta f = f_0 - f_s = C_1 K (\epsilon' - 1) \quad (3-6)$$

$$\Delta \left(\frac{1}{Q} \right) = \frac{1}{Q_s} - \frac{1}{Q_0} = C_2 K^2 \epsilon'' \quad (3-7)$$

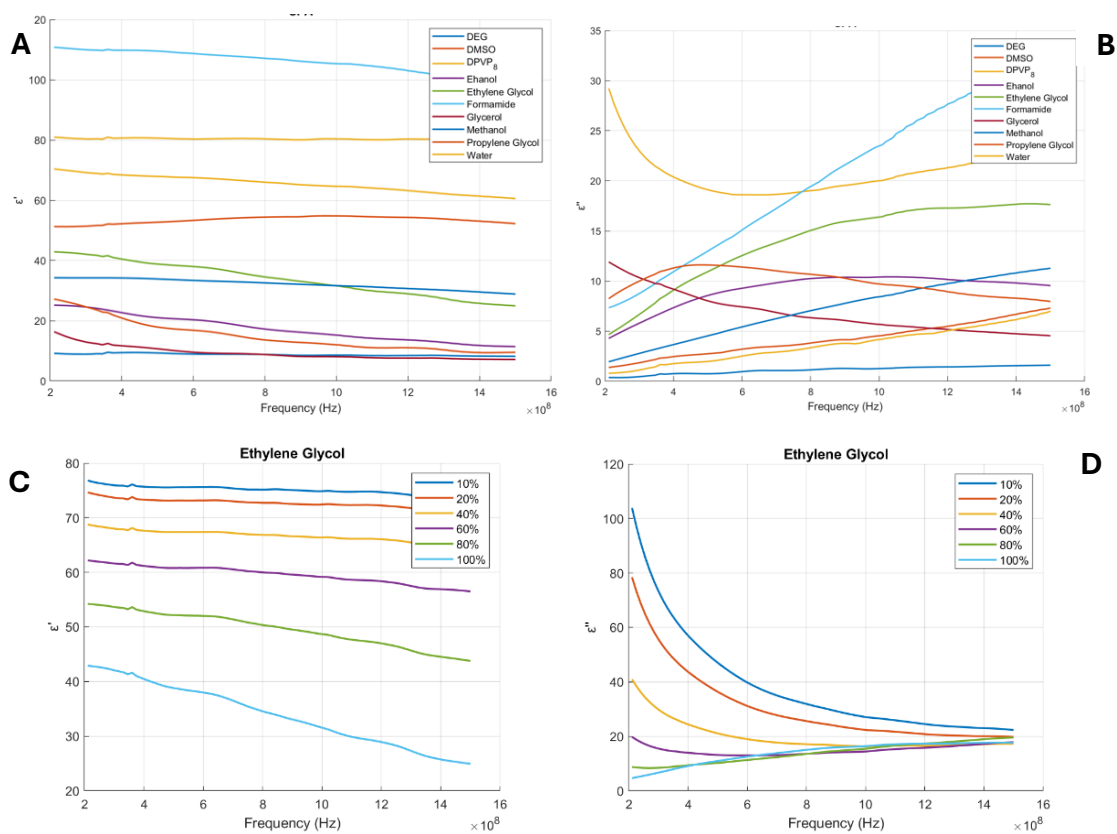
The cavity perturbation measurement system was calibrated by extracting the dielectric property at 434 MHz from the coaxial cable measured results shown in Table 3-5. Then the data will be used to know the shape factor K when reaching the highest coefficient (R^2) of determination with linear regression model.

TABLE 3-5. CALIBRATION MATERIAL FOR CAVITY PERTURBATION METHOD

Name	ϵ'_r	ϵ''_r
Methanol	34.11	3.97
Ethanol	21.93	7.83
Glycerol	15.88	13.02
Formamide	109.79	11.68
Water	80.73	1.85
Propylene Glycol	19.57	11.53
DMSO	48.06	2.3

3.6.3 Results and discussion

Figure 3-10 shows the dielectric properties of pure and binary solutions at room temperature using the coaxial probe test. Figure 3-10A and 9B show the common CPA dielectric constant and loss. The dielectric constant tends to be stable over 200 MHz to 1.5 GHz. The dielectric loss of DEG tends to increase with increasing frequency. DPVP is the common vitrification solution we used. The dielectric loss of DPVP tends to be small at around 400 MHz. Thus, it might not be the optimal solution in absorbing EM energy.



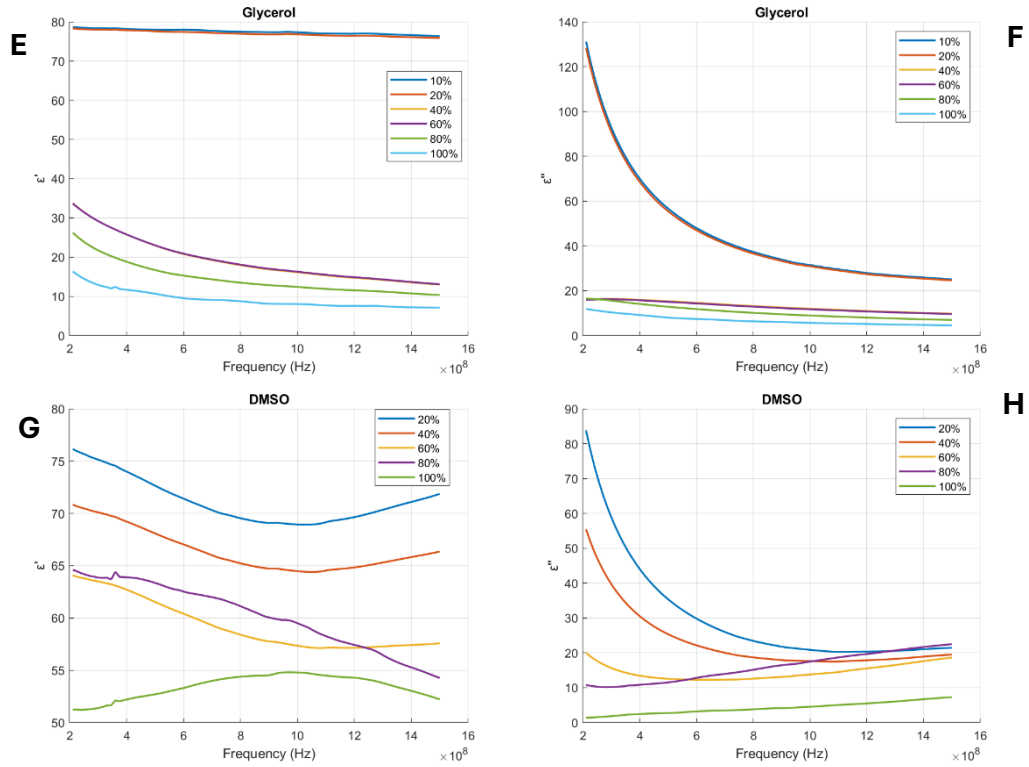


Figure 3-10. Dielectric properties of pure and binary CPA solutions at room temperature

However, the coaxial cable method has an operation temperature limit, and the resonant cavity is used in cryogenic temperature at 434 MHz. The calibration results show in Figure 3-11 and both R^2 are close to 0.99 when the shape factor, K , set to $1 / (\epsilon' + 8)$.

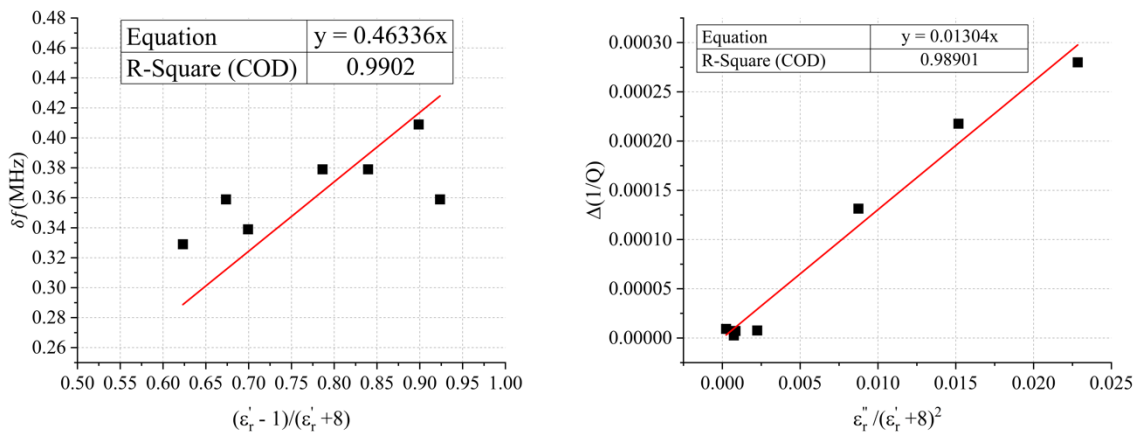


Figure 3-11. Determination of shape factor and calibration of the cavity perturbation method

Figure 3-12 shows the dielectric constant and loss. Both dielectric constant and loss present up-down trend. The starting point of dielectric increase presents a relationship to the glass transition temperature. The lower T_g , the lower the temperature starts to increase the dielectric constant, which might result from the happening of devitrification process. The unstable of dielectric constant and loss might results from the recrystallization process.

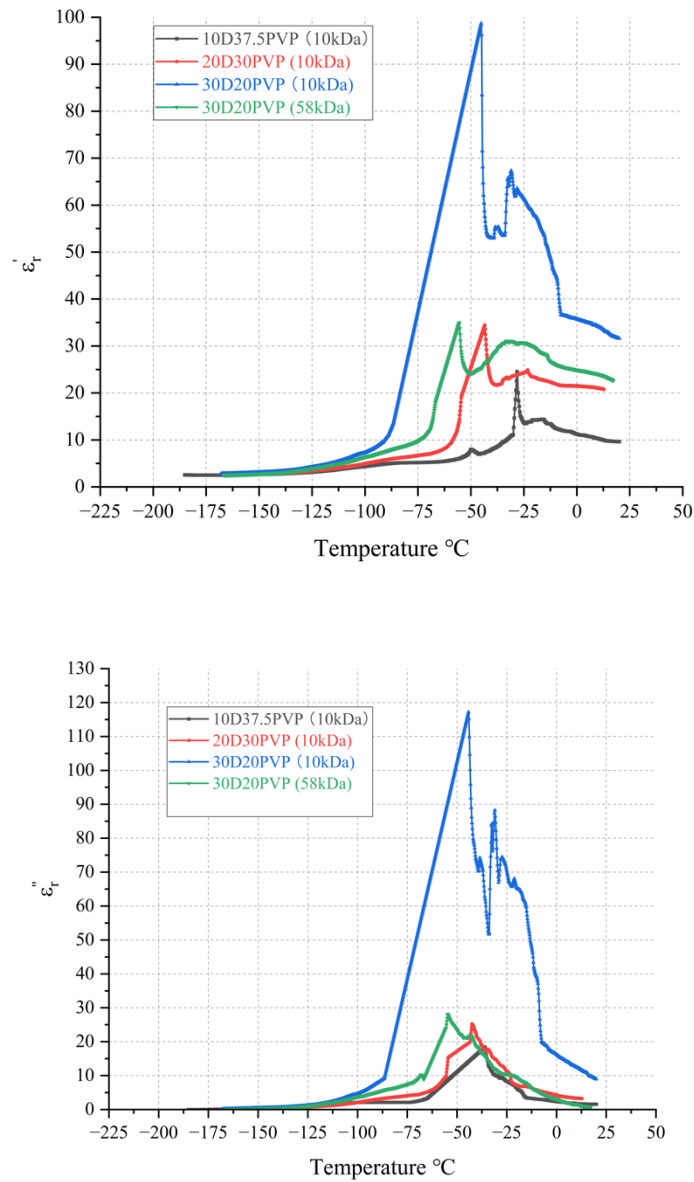


Figure 3-12. Dielectric constant (top) and loss (bottom) of vitrification solutions

Later, tangent loss of vitrification solution was calculated as it determines the ability of converting sample stored EM energy to thermal energy. Figure 3-13 shows the Tangent loss for the proposed CPAs discussed in Table 3-1. However, although sample area presents inverted-U shape, it might from the devitrification and recrystallization. Further study of the vitrified sample without recrystallization when above T_g should be investigated.

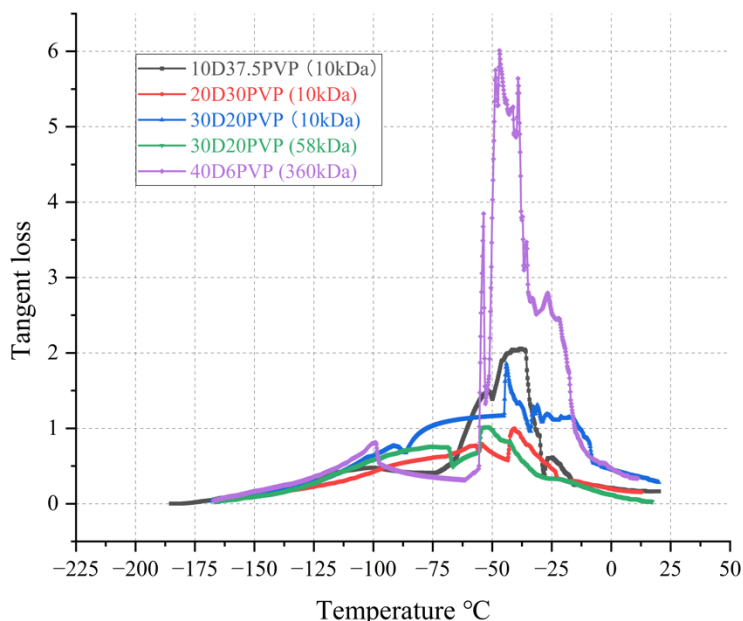


Figure 3-13. Dielectric constant (top) and loss (bottom) of vitrification solutions

3.7 Determination of biocompatibility

3.7.1 Toxicity Injury Measurement of PVP and DMSO

The biocompatibility test was conducted by using different concentrations of the PVP and DMSO and PVP mixture (DPVP) with Jurkat cell ($n = 8$). The PVP molecular weight is 10 kDa for higher solubility and easy transfer. Table 3-6 illustrates the concentration used in 96 well plate. After adding the PVP and DPVP followed by 10 μ L alamarBlue (ThermoFisher, US), the 96 well plate were placed in 37 °C incubator for 3 days. The absorbance reading was conducted at 570nm

and 600 nm (reference) by using Microplate reader (Infinite M Plex, Tecan, Switzerland) in 3.5hr, 18hr, 24hr and 42hr.

TABLE 3-6. BIOCOMPATIBILITY STUDY OF PVP AND DPVP

Row	PVP group (%)	DPVP group (%)
B	37.5	10D + 37.5P
C	18.75	5D + 18.75P
D	9.38	2.5D + 9.38P
E	4.69	1.25D + 4.69P
F	2.34	0.625D + 2.34
G	1.17	0.3125D + 1.17

3.7.2 Results and discussion

Figure 3-14 shows metabolism activity test by almarBlue from row B to row G (up to down) in 96 well plate. With increasing PVP concentration, metabolism activity is slowed but still exists. However, when 10% DMSO is introduced, there is no metabolism activity, which means the cell loses its functionality or dead. Figure 3-14 shows the absorbance reading of the two groups.

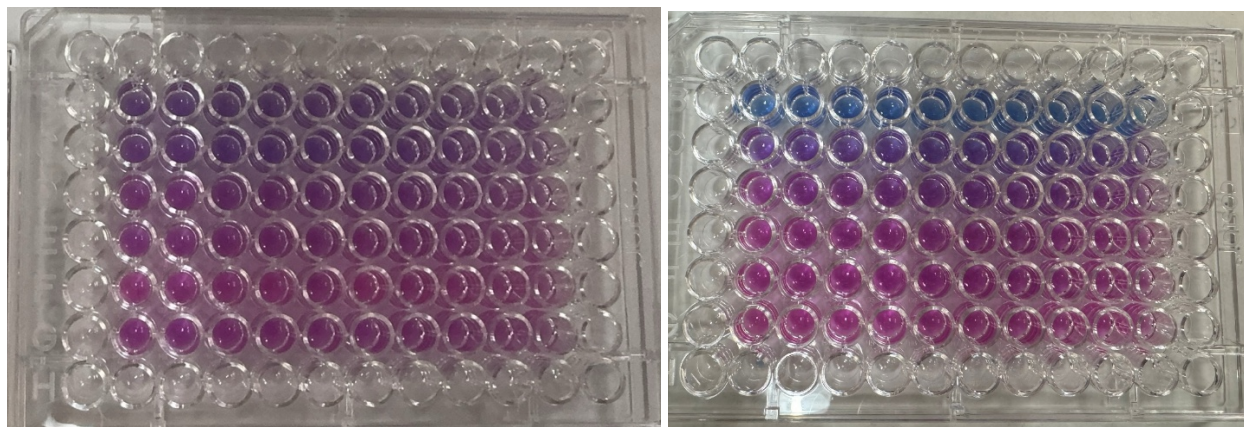


Figure 3-14. alamar Blue Test of PVP group (left) and DPVP group (right). The concentration of each row is listed in Table 3-6

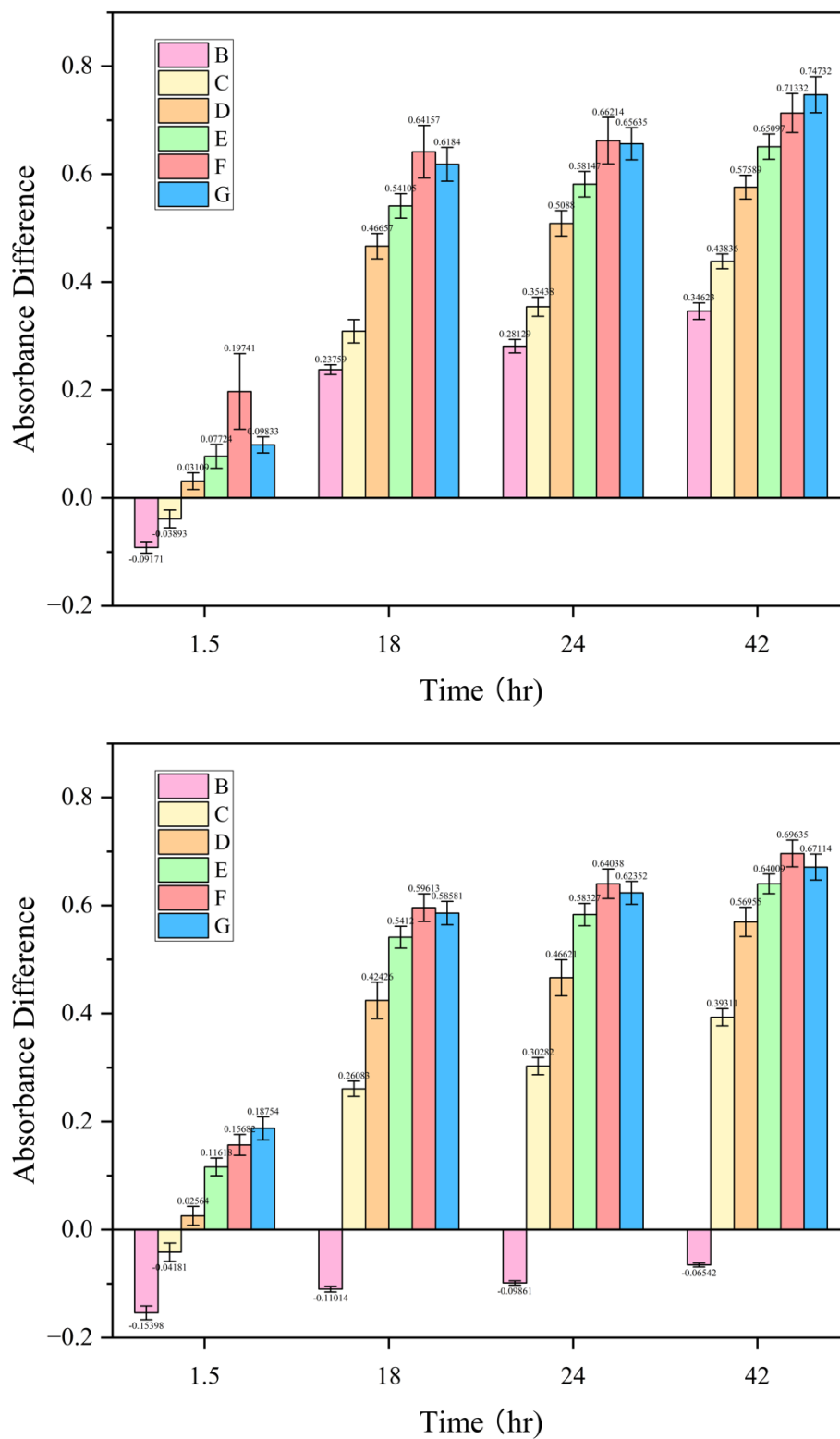


Figure 3-15. Absorbance difference in various time for 10 kDa PVP (up) and DPVP (bottom) group

From Figure 3-13 and 3-14, with the 37.5% PVP slows the metabolism activity, which might be due to the high viscosity. However, since the 18 hr, it shows similar increase compared to the rest of group, which means the cell is still alive. The DPVP group is averagely lower than the PVP group, which explains the DPVP group has more toxicity and osmolarity. In addition, the cell can't tolerate 10% DMSO group as the almarBlue keeps blue and shows no value in 570nm absorbance measurement.

3.8 Summary

In this chapter, we proposed several vitrification solutions with lower concentrations of DMSO combined with various concentrations and molecular weights of PVP.

The solution was designed by analyzing the possibility in various aspects, including glass transition temperature, viscosity, osmolality, dielectric property for future coupling with EM rewarming, and its biocompatibility with Jurkat cells. The high concentration of PVP increases the glass transition temperature and the viscosity. The PVP suppresses the metabolic activity, which might be due to the high viscosity. In summary, the high concentration of PVP provides a solution to a large volume of vitrification, and the CPA and the 10D37.5PVP have potential in the vitrification of large volume cell suspension, and the others may be used for tissue or organ cryopreservation.

Chapter 4. IMPROVED CRYOPRESERVATION OF JURKAT CELL SUSPENSIONS USING A NOVEL CPA IN FREEZING AND VITRIFICATION CONDITIONS

4.1 Introduction

The previous chapters discussed the design and development of the CPA with introducing the different molecular weight and concentration of the PVP. The last chapter focused on the vitrification tendency and the biocompatibility. However, cell suspension in cryopreservation was not employed. In the past, cell cryopreservation followed Peter Mazur's Two Factor Hypotheses described in Chapter 1, which requires cell type dependent optimal cooling rate. Vitrification was common applied to tissue as it can tolerate much higher concentration of CPAs [28, 139], however, cell vitrification is only viable for micro-liter sample such as embryo [140] and oocyte [7].

Currently, although ice inhibitors and anti-freezing proteins are continuously developing trying to enlarge the vitrification volume [122, 124], the vitrification solution still required high concentration of the permeating CPAs such as DMSO, EG, glycerol [141].

In this chapter, permeating CPA (DMSO) at much lower concentration (10% m/m %) was used to the final concentration. The novel CPA also contains 37.5% PVP (10 kDa), which has proved the vitrification ability in Chapter 3. Since the high viscosity reduces the diffusion rate of the DMSO [142], multiple CPA loading methods were tested. 4 mL of Jurkat cells with 10% DMSO and novel proposed CPAs were used, and samples were cooled to LN₂ temperature after CPA loading. The samples were rewarmed with 37 °C non-shaking water bath after stored for 2 days. The recovery rate and post-thaw biological functionalities of cells were evaluated.

4.2 Material and methods

4.2.1 Sample preparation

Jurkat cell, a cell line of human T lymphocyte cells, was chosen as the biological sample due to its importance in acute T cell leukemia, T cell signaling, HIV research, and other clinical studies. The original Jurkat cell lines were purchased from ATCC (American Type Culture Collection, Manassas, VA), then reproduced to passage 3, and cryopreserved at -196 °C. All of the cells used in this test were thawed with a water bath and cultured with the following procedures.

As an immortalized cell line, Jurkat cells were cultured in an incubator (VWR International, Radnor, PA) that set at 37 °C, 5% carbon dioxide, and proper humidity. T25 (Corning Inc., Corning, NY) flasks were used during the culture process. A unit of the cell growth medium was prepared of 450 mL RPMI medium (ATCC, Manassas, VA), 50 mL fetal bovine serum (ATCC, Manassas, VA), and 5 mL Penicillin-Streptomycin (ATCC, Manassas, VA).

The Jurkat cells were cultured to the live cell concentration around $1.24 \times 10^6 \text{ mL}^{-1}$, then aliquoted 2 mL to 4 mL cryovial for DMSO only group and 1 mL to 4 mL cryovial for DPVP group on ice for later use ($n = 6$). The viability and live-cell concentration were determined by the Trypan Blue solution (ThermalFisher, Waltham, MA).

4.2.2 CPA solution

Three groups of CPAs Stock solutions were prepared in the same day as the CPA addition.:

Group1 (DMSO only): The stock solution (20% (m/m) DMSO) was added to the cell suspension slowly with a dropwise method on the ice until the final volume reached 4 mL. Then, final concentration reaches 10% (m/m) dimethyl sulfoxide (DMSO) in CPA and Jurkat cell suspension mixture. The solution was placed on ice for 3min before transferring to LN₂ tank.

Group2 (1step_10D37.5P): The stock solution (13.3% (m/m) DMSO and 50 % (m/m) Polyvinylpyrrolidone (PVP) were added to the cell suspension slowly with a dropwise method on the ice until the final volume reached 4 mL. Then, final concentration reaches 10% (m/m) DMSO and 37.5% (m/m) PVP in CPA and Jurkat cell suspension mixture. The sample was placed on ice for 15min to allow DMSO diffusion before transferring to the LN₂ tank.

Group3 (2step_10D37.5P): First, add 26.6% (m/m) DMSO to cell suspension slowly with dropwise method on ice until it reaches to 2mL. Then, wait 3 min. Later, draw 1 ml cell suspension and add 3ml stock solution (50 % (m/m) Polyvinylpyrrolidone (PVP)) until it reaches to 4 mL. Then, shake the solution gently to uniform the mixture and place on ice 3 min before transferring to the LN₂ tank. The final concentration reaches 3.325% (m/m) DMSO and 37.5% (m/m) PVP in CPA and Jurkat cell suspension mixture.

4.2.2 Cooling device and process

Samples (n =3) of each group were transferred and plugged into the LN₂, fully immersed to ensure it beyond the optimal cooling rate, 1 °C/min.

4.2.3 Rewarming methods

The samples were transferred from the LN₂ tank to 37 °C water bath after 2 days storage. The water bath is at non-shaking condition and the sample was picked from water bath until thermal couple reading reached 0 °C, and no obvious ice crystal was observed.

4.2.4 Temperature monitoring

In the bath warming, an optical fiber was inserted into the center of cell suspension to record the temperature profile and temperature gradient within the sample.

4.2.5 Recover rate of the Jurkat cells

Cell membrane integrity was determined by Trypan Blue assay to calculate the recovery rate of the Jurkat cell. 10 μ L post-thawed cell suspension was mixed with 10 μ L staining solution and then added to the counting slide. The cell counter (Countess 3FL, Invitrogen, Carlsbad, CA) was used to obtain the number of live cells and dead cells. The recovery rate of the Jurkat cells was determined by:

$$\text{Recovery Rate (RR)} = \frac{\text{\# of live cells after cryopreservation}}{\text{\# of live cells before cryopreservation}} \times 100\% \quad (4-1)$$

4.2.6 Assessment of the post-thaw biological metabasin

The metabolic activity of the post-thawed Jurkat cell was determined by alamarBlue assay. As illustrated in Figure 4-1, 100 μ L post-thawed cell suspension of each warming method was added to the 96-well plate. The $1 \times 10^6 \text{ mL}^{-1}$ of fresh cells (positive control) was added as set it's a standard as the concentration is cell line and it is hard to align the cryopreserved cell concentration with unfrozen cell suspension even with dilution. Therefore, all cells are compared to the unfrozen cell at the cell line. The growth media was also added as negative control group. Then, 10 μ L alamarBlue assay was added to each well. The loaded plate was incubated at 37 $^{\circ}\text{C}$ for 2 days. The absorbance of each well was determined by the microplate reader (Infinite M Plex, Tecan, Switzerland) at 570 nm and 600 nm. The average absorbance readings of each group were normalized to the fresh control group and presented in the percentage.

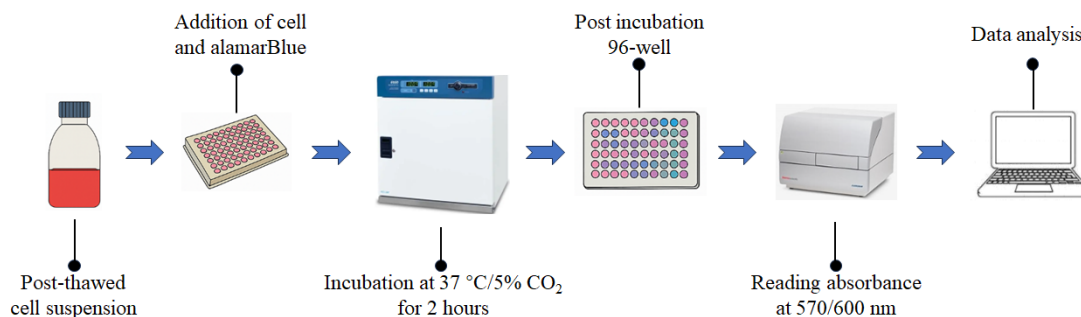


Figure 4-1. Determination of the metabolic activity of the post-thawed Jurkat cells

4.3 Results and discussion

4.3.1 Determination of the recovery rate of the Jurkat cells

The membrane integrity of the Jurkat cells with different CPA composition was tested by Typan Blue staining solution ($n = 12$). As shown in Figure 4-2, the recovery rate of Jurkat cells of the group 1, group2 and group3 were $17.55 \pm 1.77\%$, $48.73 \pm 8.65\%$, and $66.72 \pm 3.86\%$, respectively. The mean values $\pm$ standard deviations were determined based on twelve replicates ($n = 12$).

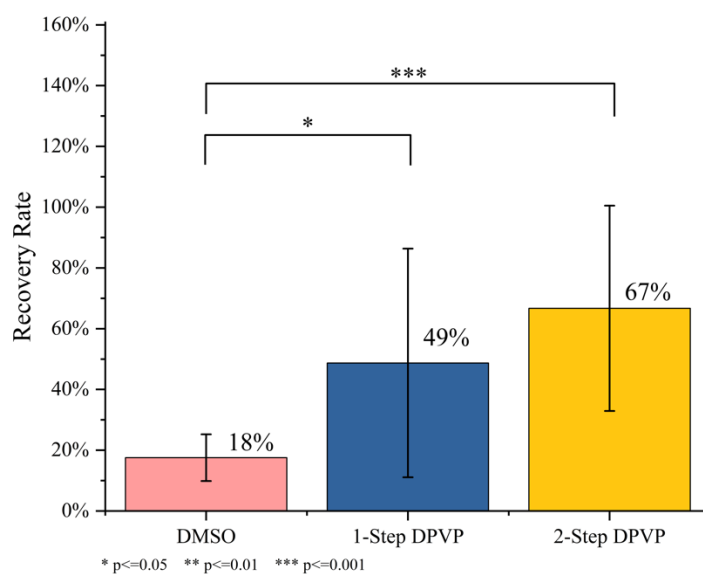


Figure 4-2. The recovery rate of the post-thawed Jurkat cells

The utilization of PVP accomplished vitrification in LN₂ cooling. As a result, the recovery rate of Jurkat cells using 2 step_10D37.5P was increased by 49.17% from DMSO only group ($P = 7.36 \times 10^{-5}$), and the 1 step_10D37.5P increase 31.78% from DMSO only group ($P = 0.0102$). From Figure 4-2, although the 2step group faces the highest DMSO concentration initially, it presents the best recovery rate, which prove the effectiveness of the proposed novel CPA and 2 step CPA loading method.

4.3.2 Determination of the metabolic activity of the post-thawed cells

As an immortalized cell line, a defining function of viable Jurkat cells is their proliferation rate or metabolic activity. The post-thawed Jurkat cells were incubated at 37 °C with 5% carbon dioxide, the metabolic activity ($n = 16$) was assessed by alamarBlue assay after 2 days as the cell concentration is low right after rewarming (diluted 8X before freezing for 2 step and 4X for 1step group). The metabolic activity of viable cells indicated by a change from resazurin (blue color) to resorufin (pink color) through the reduction reaction of active living cells. Figure 4-3 represents the color change of Jurkat cells with different CPA groups over time. In each 96-well, from left to right, the cells were from the 10%DMSO, 1 step_DPVP, 2 step_DPVP and the positive control. Negative control was in another plate, not shown in the photo.

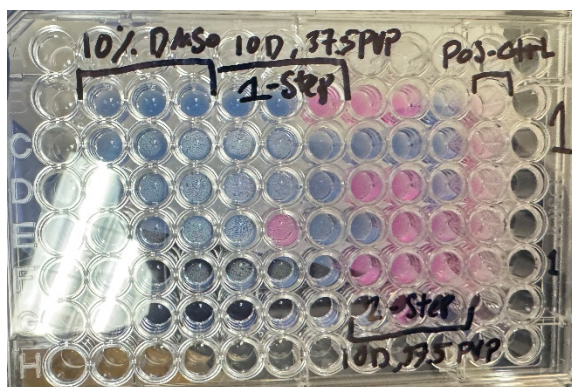


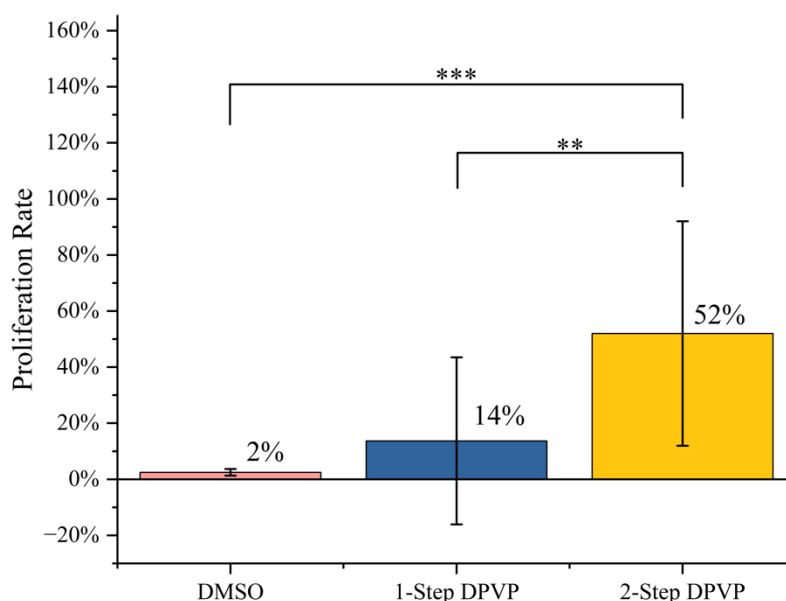
Figure 4-3. Incubating the cryopreserved and rewarmed cell

All groups remain blue until the second day. The 1 step and 2 step DPVP group starting to be purple and pink color. A confirmatory low metabolic activity was observed in the negative control group as the samples stayed blue color for all of the testing days. The quantified absorbance readings were obtained by the microplate reader and calculated by the below equation.

$$Relative\ Viability = \frac{A_{\square}^{sample} - A_{\square}^{negative}}{A_{\square}^{positive} - A_{\square}^{negative}} \times 100\% \quad (4-2)$$

Where the A is the absorbance difference between the 570nm and 600nm.

Figure 4-4 shows the metabolism or proliferation rate after rewarming. The mean values $\pm$ standard deviations were determined based on sixteen replicates (n = 16).



* p<=0.05 ** p<=0.01 *** p<=0.001

Figure 4-4. Determination of the post-thawed cells metabolic activity by alamarBlue

DMSO group does not turn to pink across all sixteen samples while 2 out of 16 turns to pink in the 1-step DPVP group and the average proliferation rate is 14% compared to fresh control at cell line concentration. Although the concentration at initial was diluted 2X compared to other

2 groups, most of 2step_DPVP turns to pink color after 2 days, which concludes the 2 step DPVP preserve both the cell membrane integrity and the functionality.

After two days of incubation, the average metabolic activity achieved by DMSO, 1step_DPVP, and 2step_DPVPs were $2.49 \pm 1.16\%$, $13.70 \pm 28.82\%$, and $52.00 \pm 38.75\%$, respectively. Figure 4-4 shows the P value between DMSO and 1step_DPVP and 2step_DPVP. The variance may be due to the high viscosity of the DPVP solution and recrystallization in the core area during thawing. The results conclude the potential of large cell suspension vitrification and warmed with water bath.

4.4 Conclusion

In this chapter, 10% DMSO + 37.5% PVP was utilized to achieve vitrification of large volume (4 mL) of cell suspension in LN₂. Both the recovery and proliferation rate show a significant difference between the DMSO and DMSO+PVP group by using waterbath as rewarming method. The novel CPA led to the better biological characteristics of the post-thawed Jurkat cells. The addition of PVP is almost fourth time better in the recovery rate of Jurkat cell ($66.72 \pm 3.86\%$) compared to the DMSO only group when directly plugged into the LN₂ tank and rewarmed by convective water bath method and presented a high metabolic activity to the fresh control group after two days of incubation.

The use of PVP significantly reduced the required concentration of DMSO to approach vitrification and bring a potential transformation the convention cooling of cell suspension to a new era of cell vitrification. By having that, the optimal cooling of cells is no longer needed, and the cell suspension cryopreservation is not limited to single cell type anymore, which brings a great future to cellular therapy. In addition, the vitrification solution also potentially modifies to use in tissue and organ cryopreservation. Lastly, by observing the rewarming process, the large volume

of cell experiments may have more severe recrystallization, and the water bath doesn't provide sufficient rewarming rate. Therefore, powerful volume metric heating is needed.

Chapter 5. DEVELOPMENT AND OPTIMIZATION OF A BROADBAND SINGLE-MODE ELECTROMAGNETIC RESONANCE CAVITY INTEGRATED WITH AUTO-RESONANT TRACKING

5.1 Introduction

The study of the vitrification tendency of the vitrification solution is discussed in Chapter 3. However, it has been proved the water bath is not ideal to rewarming large volume of cryopreserved samples, due to its low rewarming rate and the surface convection heat transfer mechanism. As discussed in Chapter 1, Dielectric heating using electromagnetic wave is promising for rewarming the cryopreserved sample volumetrically. From equation 1-5, the dielectric heat generation rate per unit volume is proportional to the frequency, dielectric property, and electric field intensity. The frequency and dielectric property define the penetration depth (δ) of material govern by [143]:

$$\delta = \frac{c}{2\pi f \sqrt{\frac{\epsilon'}{2} \left[\sqrt{1 + \left(\frac{\epsilon''}{\epsilon'}\right)^2} - 1 \right]}} \quad (5-1)$$

Another important parameter is the electric field intensity, which is not relied on the material. Therefore, single mode electromagnetic resonant cavity (SMER) is selected to concentrate the electric field in a designated area. Chapter 1.3.3.2(VI) describes the history and development of the SMER system by Luo, Pan and Ren. However, only 434 MHz cavity was discussed, and the broadband frequency and systematic cavity design protocol were missing. In addition, although the SMER system is promising in a 25 mL sample, future development is needed to further improve the resonant tracking by adjusting the incident frequency. Otherwise,

the resonant status is disrupted, and the electric field cannot be focused. This adjusting method is different from the capacitor tuning described in the capacitor heating section where the SMER system tends to adjust the frequency in tuning the system load impedance to achieve resonant/impedance matching.

In this chapter, we majorly focus on systematic development of the SMER system in broadband frequency for different uses. In addition, we worked on optimizing previously developed systems in reducing the power loss. More importantly, the auto-resonant tracking system was built to track the temperature dependent resonant frequency to ensure the max electric field intensity.

5.2 Theory of single mode resonant cavity design

Dielectric heating utilizes the EM wave as a heating source to achieve volumetric heating. Thus, understanding the propagation of EM waves is important. Appendix I includes the wave equation of parallel waveguide, rectangular waveguide, and resonant cavity. The single mode was utilized in the following design since the electric field in a multi-mode cavity is hard to predict and non-uniform, which brings difficulty in control and causes thermal runaway effect. Figure 5-1 shows the geometry of the parallel waveguide (A), rectangular waveguide (B), reflected cavity (C), and resonant cavity (D) and its boundary condition.

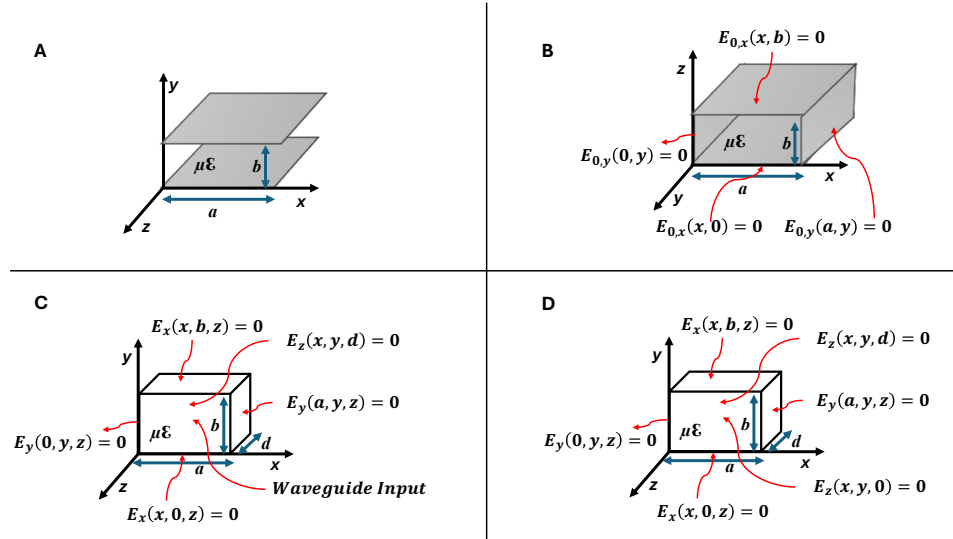


Figure 5-1. Geometry of the parallel waveguide (A), rectangular waveguide (B), reflected cavity (C), and resonant cavity (D) and its governing boundary conditions.

To excite a single standing wave inside the cavity (single mode), the dominate mode concept is crucial. For any size cavity, it has a cut-off frequency and resonant frequencies for a variety of modes. When the input frequency is below the cut-off frequency (first mode), no mode will be excited in the cavity. When the frequency is above the secondary mode frequency, two or more modes will possibly exist in the cavity. Thus, the desired frequency should be larger than the cut-off frequency and also lower than the secondary mode at the designed cavity. The governing equation of the cut-off frequency is shown when air is filled media inside the cavity:

$$f_c = \frac{1}{2\sqrt{\epsilon\mu}} \sqrt{\left(\frac{m}{a}\right)^2 + \left(\frac{n}{b}\right)^2} \quad (5-2)$$

Where f_c is the cutoff frequency of the waveguide, ϵ and μ are the material permittivity and permeability, m and n are the mode number, determining the number of half wavelength in x and y directions shown in Figure. 5-1.

Since TE mode presents maximum electric field intensity at the center of the rectangular shown in Figure 5-2.

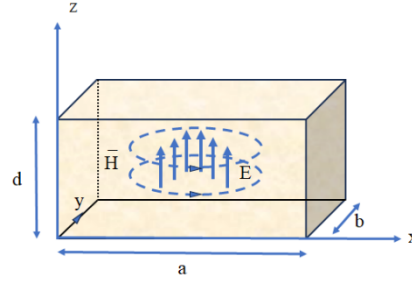


Figure 5-2. Electric and magnetic field of TE₁₀₁ mode. The electric field is focused at the center [144]

Thus, we use TE mode as an example to design the TE₁₀₁ and TE₁₀₂. However, TM mode will follow the same process. The governing equation of the TE mode in a resonant cavity is:

$$f_{mnp}^{TE} = \frac{c}{2} \sqrt{\left(\frac{m}{a}\right)^2 + \left(\frac{n}{b}\right)^2 + \left(\frac{p}{d}\right)^2} \quad (5-3)$$

Where f_{mnl}^{TE} is the resonant frequency when the filling medium inside of the cavity is air and c is the light speed at vacuum space. m, n, p are the mode numbers (non-negative integers), which represent the number of the half wave in the corresponding direction. m and n cannot be both equal to zero and p is larger or equal to 1. a, b, d are the cavity dimensions in x, y, z direction shown in Figure 5-2.

5.2.1 Development of the TE₁₀₁ resonant cavity

The governing equation of the electric and magnetic field in TE₁₀₁ mode cavity are shown below and E_z , E_x and H_y equals to 0:

$$H_z = -j2AC \cos(k_x x) \sin(k_z z) \quad (5-4)$$

$$H_x = \frac{j2ACK_z k_x \sin(k_x x) \cos(k_z z)}{k^2 - k_z^2} \quad (5-5)$$

$$E_y = \frac{-2AC\omega\mu k_x \sin(k_x x) \sin(k_z z)}{k^2 - k_z^2} \quad (5-6)$$

The dominate mode concept is crucial to excite a single standing wave inside the cavity (single mode). For any size cavity, it has a cut-off frequency and resonant frequencies for a variety of modes. When the input frequency is below the cut-off frequency (first mode), no mode will be excited in the cavity. When the frequency is above the secondary mode frequency, two or more modes may exist in the cavity. Thus, the desired frequency is 434 MHz or 915 MHz, it should be larger than the cut-off frequency and also lower than the secondary mode at the designed cavity. From Eq(5-2), we noticed the potential dominance in a rectangular cavity is TE101, TE011, or TE110, so we need to take those into consideration when designing the TE101 mode resonant cavity.

TABLE 5-1. LIST OF POTENTIAL DOMINANT MODE AND ITS RESONANT FREQUENCY AT A RECTANGULAR CAVITY

Potential Mode	TE101	TE011	TE110
$(\frac{2f_{mnp}}{c})^2$	$(\frac{1}{a})^2 + (\frac{1}{d})^2$	$(\frac{1}{b})^2 + (\frac{1}{d})^2$	$(\frac{1}{a})^2 + (\frac{1}{b})^2$

From Table 5-1, the relationship below is built to ensure that TE101 is the dominant mode.

$$(\frac{1}{a})^2 + (\frac{1}{d})^2 < (\frac{1}{b})^2 + (\frac{1}{d})^2 \quad \& \quad (\frac{1}{a})^2 + (\frac{1}{d})^2 < (\frac{1}{a})^2 + (\frac{1}{b})^2 \quad (5-7)$$

Eq (5-7) can be simplified as

$$(\frac{1}{a})^2 < (\frac{1}{b})^2 \quad \& \quad (\frac{1}{d})^2 < (\frac{1}{b})^2 \quad (5-8)$$

Since the d is the propagation direction, it is the longest side of the cavity. Eq (5-8) becomes $b < a < d$. For the TE₁₀₁ mode, both a and d should be positive numbers to satisfy the physical geometry. Thus, with the desired resonance frequency, the minimum a and d should be governed by:

$$a > \frac{c}{2f_{101}} \quad \& \quad d > \frac{c}{2f_{101}} \quad (5-9)$$

Since $d > a$, the minimum d value is governed when $a = d$:

$$d > \frac{\sqrt{2}c}{2f_{101}} \quad (5-10)$$

5.2.2 Development of the TE₁₀₂ resonant cavity

As discussed in Chapter 1, nanowarming has been successful in rewarming cryopreserved samples using the magnetic field. The resonant cavity in TE mode can utilize the electric and magnetic fields in the designated mode. TE₁₀₂ is extended from the TE₁₀₁ mode but has a full wave in the propagation direction. Thus, there will be a magnetic field concentrated area at the center of the cavity. Table 5-2 lists all potential modes during the design of the TE₁₀₂ cavity.

TABLE 5-2. LIST OF POTENTIAL DOMINANT MODE AND ITS RESONANT FREQUENCY AT A RECTANGULAR CAVITY

Potential Mode	TE ₁₀₂	TE ₀₁₁	TE ₁₁₁ or TM ₁₁₁	TE ₀₁₂	TE ₀₂₂	TM ₁₁₀
$(\frac{2f_{mnp}}{c})^2$	$(\frac{1}{a})^2 + (\frac{2}{d})^2$	$(\frac{1}{b})^2 + (\frac{1}{d})^2$	$(\frac{1}{a})^2 + (\frac{1}{b})^2 + (\frac{1}{d})^2$	$(\frac{1}{b})^2 + (\frac{2}{d})^2$	$(\frac{2}{b})^2 + (\frac{2}{d})^2$	$(\frac{1}{a})^2 + (\frac{1}{b})^2$

Similar to the process in section 5.2.1, the TE₁₀₂ should be lower than all the modes listed in Table 5-2. Then, we can find the relationship below:

$$d > a > b \quad \& \quad d > 2b \quad (5-11)$$

a and d still need to be positive, and d should be larger than a, but the $m \neq p$, so the governing length for a and b will be different, shown as:

$$a > \frac{c}{2f_{102}} \quad \& \quad d > \frac{\sqrt{5}c}{2f_{102}} \quad (5-12)$$

With given d and a, the b value, the b length is determined by the desired bandwidth between the TE₁₀₂ and TE₀₁₁, which shows the following:

$$\frac{2(f_{011} - f_{102})}{c} = \sqrt{\left(\frac{1}{b}\right)^2 + \left(\frac{1}{d}\right)^2} - \sqrt{\left(\frac{1}{a}\right)^2 + \left(\frac{2}{d}\right)^2} \quad (5-13)$$

5.2.3 Critical rewarming rate guided maximum sample volume in a given cavity

For a homogeneous sample, the heat required to increase per degree Celsius per unit volume can be found as:

$$q = \rho C_p \frac{dT}{dt} \quad (5-14)$$

Where the ρ is the density of the material, C_p is the specific heat at constant pressure, and the dT/dt is the temperature change rate.

Eq(5-14) can be connected to Eq(1-5) and find the required minimum of electric field intensity inside the cavity during the rewarming process by applying the critical cooling rate equal

to dT/dt and selecting the minimum dielectric loss during the thawing temperature range, shown as follows:

$$E_{min}^2 = \frac{\rho C_p CRR}{2\pi f \varepsilon_{r,min}'' \varepsilon_0} \quad (5-15)$$

Where the E_{min} is the minimum required electric field; CRR is the critical rewarming rate; and $\varepsilon_{r,min}''$ is the minimal dielectric loss of material, which is usually at the lowest temperature.

Although the resonant frequency of the loaded cavity always exists, no matter the sample volume or dielectric properties, the electric intensity inside of the cavity will be depressed, which can be described by the quantity of quality factors. The larger the quality factor, the stronger the electric field intensity will be. At the time, the resonance change between the loaded and unloaded cavities are described by Eq (3-4). Thus, for a given cavity (fixed power input), if the material dielectric loss and the minimum required quality factor are known, we can determine the maximum volume of the inserted sample, which avoids insufficiency in rewarming and recrystallization. Thus, finding the relationship between the quality factor and the electric field intensity is essential.

The Quality Factor, commonly referred to as the Q factor, is a dimensionless parameter that describes how underdamped an oscillator or resonator is. It characterizes the relationship between stored energy and energy dissipation in a system. Q factor can be expressed as:

$$Q = 2\pi \frac{\text{Energy Stored in Cavity}}{\text{Energy Lost per Cycle to Walls}} \quad (5-16)$$

We know the relation between the quality factor and input power is

$$E = \sqrt{\frac{8QP_{in}}{2\pi f \epsilon'_r V_c}} \quad (5-17)$$

The maximum sample volume can be found by connecting the equation (3-5) and the experiment calibration results as follows:

$$V_s = \left(\frac{1}{\rho C_p CRR \epsilon'_r V_c} - \frac{1}{Q_0} \right) \frac{V_c}{4\epsilon''_{r,min}} \quad (5-18)$$

5.2.4 Development of TE101 and TE102 resonant cavity for 900-930 MHz solid state generator

The solid-state generator (SSG) can generate the EM wave in amplitude and reduce the noise, making it a more focused output frequency. However, the adjustable range of the SSG is usually limited (900 - 930 MHz) compared to the traditional signal generator (250 kHz – 3 GHz), which limits the use of SSG when designing the resonant frequency at 915 MHz when the required frequency input decreases with sample temperature increase. By re-arrange the Eq(5-18), we can know the maximum sample volume ratio with a given material type, described as

$$\frac{V_s}{V_c} = \frac{(f_0 - f_s)}{2f_s(\epsilon'_r - 1)} \quad (5-19)$$

For example, for an unloaded cavity with a resonant frequency of 915 MHz, the maximum volume ratio we can heat for room-temperature water ($\epsilon'_r = 80$) is 0.0001037 and it about 0.89 mL when $a = 0.2\text{m}$, $b = 0.15\text{m}$ and $d = 0.286\text{m}$. Thus, we need to carefully design the resonant

frequency on the unloaded cavity with the material properties and volume. From Chapter 3.6.1 and 3.6.2, permittivity can be known. Thus, if the potential cryopreserved sample is determined and the sample dielectric constant increases with temperature increase, the unload cavity frequency should be determined with the SSG's low-temperature material properties and upper bond (930 MHz). The unloaded cavity resonant frequency can be expressed through an implicit equation shown as:

$$\frac{2V_s(\varepsilon'_r - 1)}{V_c} = \frac{f_0}{930} - 1 \quad (5-20)$$

In Eq (5-20), the V_c and f_0 are dependent, which can be solved with a two-parameter model using the fitting method.

5.2.5 Development of the TE₁₀₁ reflected cavity

Although the resonant cavity shows a high energy concentration effect, it still requires precise control to overcome the resonant frequency drift by changing material properties when temperature changes. In addition, the coaxial cable is not suitable for handling high power compared to waveguides. Thus, SSG often has a waveguide as an output port, so we can directly connect a cavity to the output port using a waveguide port, the so-called reflected cavity. The reflected cavity connects five metal walls; the remaining side is a waveguide port. The TE mode resonant frequency still follows the Eq(5-3), and the a and b are set to the waveguide port dimension. The propagating EM wave travels into the reflected cavity and is bounced back to improve the electric field intensity. Unlike the resonant cavity, it allows EM waves to oscillate inside of the cavity; the reflected cavity will reject the EM wave back to the source from the input

port. The electric field intensity can be found in superposition theory, and the field intensity will be doubled if considering the perfect conducting wall, which is shown in the following:

$$E_{tot} = E_{incident} + E_{reverse} = 2E_{incident} \quad (5-21)$$

5.3 Prototype design and optimization

We have successfully designed the 434 MHz and 915 MHz by applying the above design theory. In this section, we focus on the improvement of 434 MHz resonant cavity, experimental setups on 915 MHz reflected and resonant cavity.

5.3.1 Improvement of 434 MHz resonant cavity system design

From our research group, Luo designed the 434 MHz resonant cavity (0.40m (H) x 0.35m (W) x 0.68m (L)) [46]. Later, Pan and Ren applied the system to solutions and biological samples, including jugular tissue immersed in 25 mL of vitrification solutions. Figure 5-3 (top) shows the system design of Ren's work. The EM power is generated from the signal generator and is amplified by a power amplifier to around 500W. Then, the EM wave was transmitted to the circulator, directional couplers, and a resonant cavity via the coaxial cable (R214U). The current system has two limitations: 1) energy will be consumed by a circulator, a directional coupler (mini circuit ZGBDC20-33HP+), and 3 sections of coaxial cables; 2) The current directional coupler's maximum handling power is 250W, which causes potential damage on both the directional coupler and spectrum analyzer when using 500W power. Figure 5-3 (bottom) shows an updated system design by relocating the directional coupler to another side of the circulator. The transmitted power

$$P_t = P_{in}/(IL/10)^{10} \quad (5-22)$$

connected to a waveguide adaptor by WR975 port with a copper tape seal. The waveguide adaptor converts WR975 to a 1-5/8 EIA port with a flange connection. The 1-5/8 EIA is converted to 7/16 DIN probe antenna, which excites the EM wave inside the cavity. Figure 5-4 (right) shows a reflected cavity design as discussed in Chapter 5.2.5.

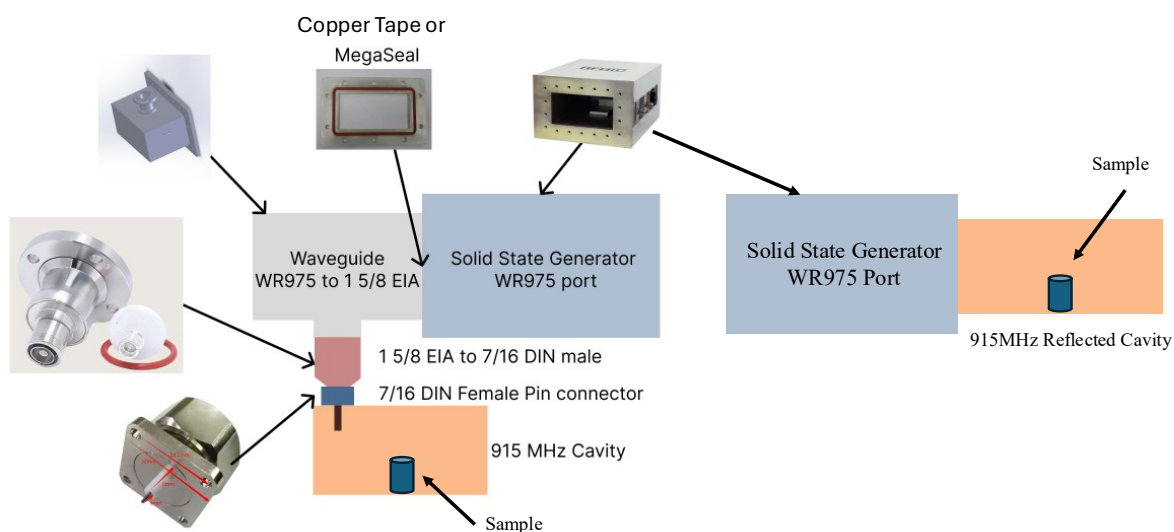


Figure 5-4. System design of the resonant (left) and reflected (right) cavity

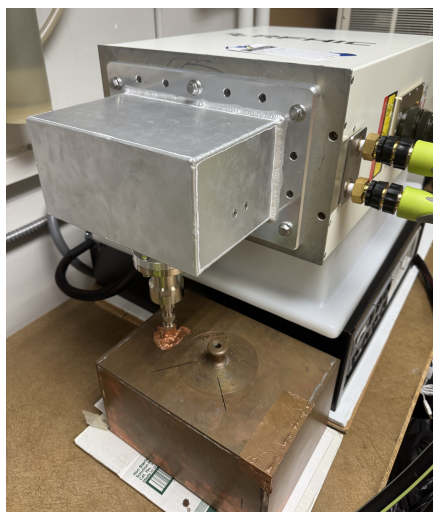


Figure 5-5. 915 MHz cavity with SSG

5.3.3 Design of auto-resonant tracking system

The resonant drift is common in resonant cavities as the material property changes with increasing temperature or phase change during the rewarming process. Without proper input resonant frequency, the cavity will not resonate and will either not form any mode (below the cutoff frequency) or multi-mode exist inside the cavity. Either of them will result in a low electric field intensity and unpredictable. Therefore, we build an auto-resonant tracking module for the SMER system. Figure 5-6 shows the flow chart of the auto-resonant tracking module, which added two RF switches and a network analyzer and replaced the directional coupler and the spectrum analyzer in Figure 5-4. The hold system was organized into a movable metal cabinet shown Figure 5-7. The operation protection and assessment and protection are described below.

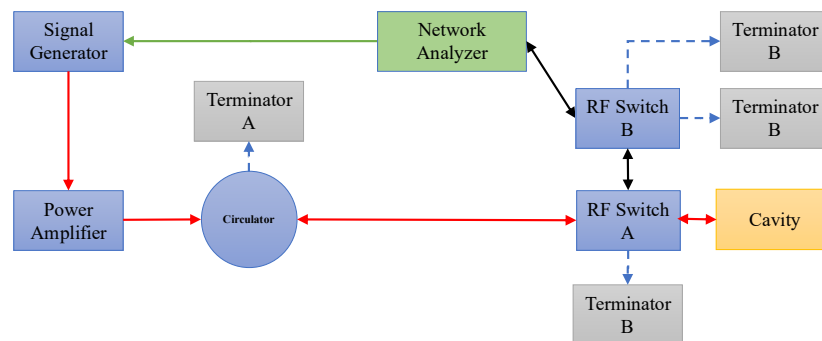


Figure 5-6. Design of RF auto-tracking system

Operation Procedure:

1. Resonant frequency measurement — RF witch B connect NA, RF switch A connect switch B – **Back solid line**
2. Send resonant frequency to signal generator — **Green solid line**
3. Switch A turn to the power meter side and switch B turn to terminator B (ns)
4. Turn on SG and Amplifier — **Red solid line**
5. Turn off the Amplifier

6. Repeat step 1 – 5

Assessment and Protection:

- **RF Switch A fault**

Case 1(Measurement mode, NA working): if RF power meter detects energy, switch A is fault.

Case 2(Heating mode, Amplifier working): RF power returned from the cavity is partially transmitted to switch B and is directed to Terminator (dummy load) B.

- **RF Switch B fault**

Case 1(Measurement mode, NA working): No resonant frequency detected. Since the amplifier is off, NA is safe.

Case 2(Heating mode, Amplifier working): No problem, all returned power will go back to the circulator and terminator A.



Figure 5-7. 434MHz SMER system with auto-tracking system included. From left to right are the signal generator, amplifier, auto-tracking system, and cavity system.

5.4 Simulation

Applying the finite element method, the numerical modeling of the electromagnetic resonant rewarming was accomplished with COMSOL Multiphysics (COMSOL Inc, Burlington,

MA). As shown in Figure 5-8, the model of the rewarming system consists of the resonant cavity, the electromagnetic power input, the coaxial transmission line, the probe antenna, and the cryopreserved sample solution. Table 3-2 presents the dimension and physical properties of the rewarming system.

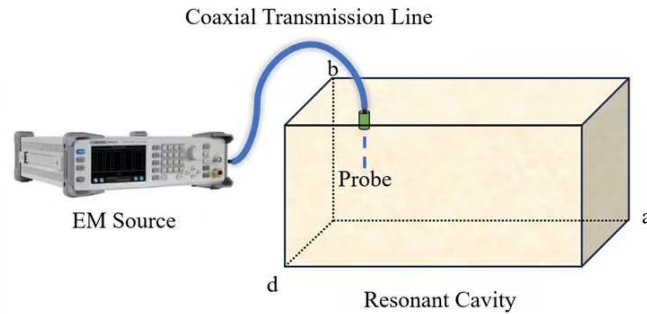


Figure 5-8. Schematic of the simulated electromagnetic resonant rewarming system

The Nyquist criterion was applied to the meshing grid of the simulation [145]. The maximum size of the element grid was constrained smaller than half of the wavelength of the electromagnetic wave. Six grids per wavelength in the finite element analysis were also employed to solve Maxwell's equation [146]. Tetrahedral grids were selected in this study due to the smaller grid size comparing to the discretization of the resonant rewarming system. Once the tetrahedron grid was applied, the grid size of the resonant chamber was calculated to be less than 10% of the wavelength of electromagnetic wave. Figure 5-9 shows the mesh preparation; different meshing approaches were adopted for different components of the system. Refined meshes were created near the boundaries of surfaces, while the area around probe antenna and central part of the sample holder were treated with enhanced meshing.

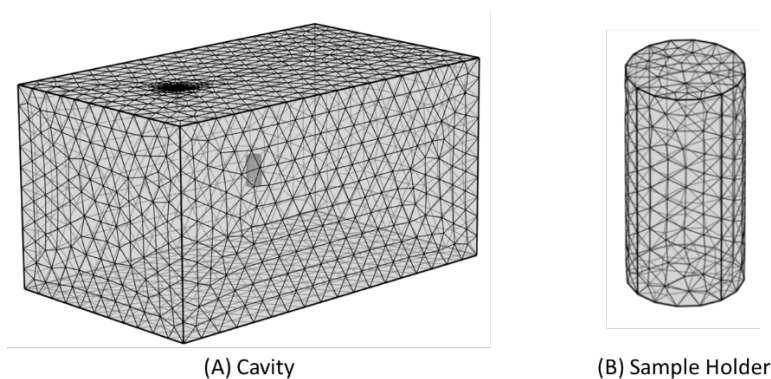


Figure 5-9. Meshed computation model of the resonant cavity

TABLE 5-3. THE DIMENSION AND PHYSICAL PROPERTIES OF THE RESONANT CAVITY SYSTEM

Parameters	Value	Unit
Length of the cavity	Frequency dependent	mm
Width of the cavity	Frequency dependent	mm
Height of the cavity	Frequency dependent	mm
Length of the probe antenna	40 (may change for best E field)	mm
Radius of the probe antenna	1.5 (may change for best E field)	mm
Coaxial transmission line impedance	50	Ω
Power input	400	W
Electrical conductivity of copper wall	5.9×10^7	Sm^{-1}
Relative permittivity of air	1	
Electrical conductivity of air	0	Sm^{-1}

5.5 Experiment

5.5.1 Validation of the auto-resonant tracking performance

30% (m/m) DMSO and 30 % (m/m) Polyvinylpyrrolidone (PVP, 10 kDa) in PBS was prepared and used as test solution. Then, the solution was added to 20 mL plastic bottom and optical fiber was placed as the center of the solution. Later, the sample was directly plugged into the LN_2 tank and vitrification was achieved by observing the transparency. Two rewarming methods were applied:

Untracking ($n = 1$): vitrified sample was placed into the center of the 434 MHz resonant cavity and measure the resonant frequency at -150°C , then RF power on at 100 W (50 dbm) until

the temperature reaches to 0 °C. Only one group was tested to prevent equipment damage from excessively reflected power due to non-resonant status.

Auto-resonant tracking (n = 3): vitrified sample was placed into the center of the 434 MHz resonant cavity. The auto-resonant tracking control system starts the initial measurement and set the current resonant frequency to signal generator; the amplifier is set to 100 W (50 dbm) at standby mode and the heat starts when temperature reaches -150 °C. The resonant frequency adjustment interval is 5 s and during the adjustment the amplifier standby time is about 1.6s. The heating stops when temperature reaches 0 °C.

5.5.2 Validation of SMER heating in vitrified sample

30% (m/m) DMSO and 30 % (m/m) Polyvinylpyrrolidone (PVP, 10 kDa) in PBS was prepared and used as test solution. Then, the solution was added to 20 mL plastic bottom and optical fiber was placed as the center of the solution. Two cooling methods were applied to achieve frozen and vitrification condition:

Frozen group: sample was directed placed and cooled by -80 °C freezer.

Vitrification group: sample was directed plugged into LN2 tank.

The rewarming was used the 434 MHz SMER system with auto-resonant tracking system at 400W (55.5 dBm). The frozen group was rewarmed from -80°C and the vitrification group was rewarmed from -150 °C to avoid the re-crystallization and extract the data beyond the -80 °C. Both rewarming are stopped when temperature reached 0 °C.

5.6 Result of cavity design

5.6.1 Development of TE101 cavity: 915MHz study example

From section Chapter 5.2.1, a , b , and c at the 915 MHz can be determined. d should be larger than 0.232 m and the relationship between a and d are governed by Eq(5-9) and shown in Figure 5-10A. In addition, the volume and cavity surface (Figure 5-10B) is study regarding to the changing length of the cavity in propagation direction when $b = a - 0.05$. Figure 5-10C-E shows the cavity surface area, quality factor and the energy dissipated over the input power. From Figure 5-10D and 5-10E, the quality factor decreases and dissipated energy increase when d increases. Thus, we want to set the d close to the 0.232m. The surface area of the cavity affects the material cost and shows minimum value when d increase. Finally, we set the cavity dimension is $0.2\text{m} \times 0.15\text{m} \times 0.286\text{m}$ ($a \times b \times d$). Copper is selected as the cavity material due to its high electric conductivity.

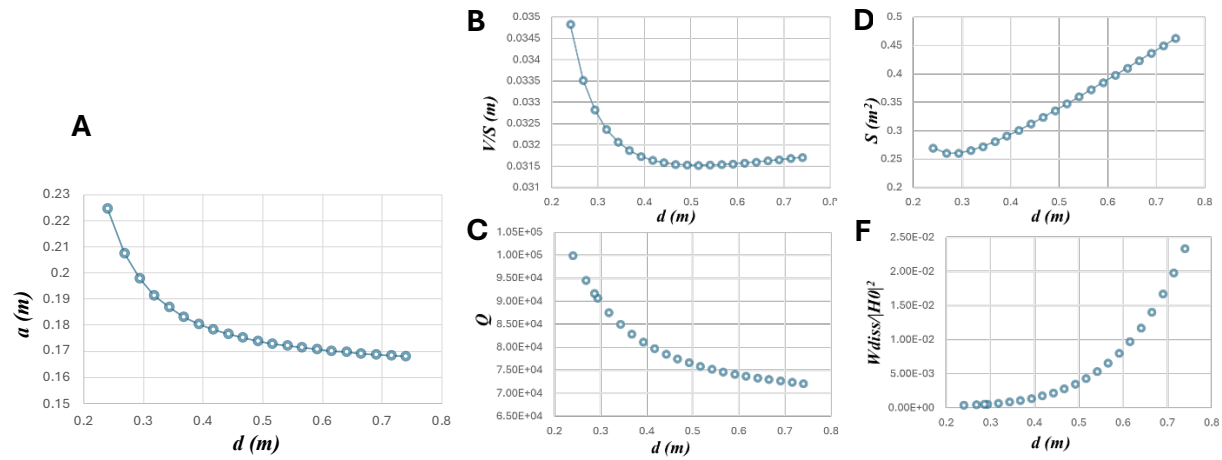


Figure 5-10. TE101 cavity design parameter. (A) Relationship between the cavity a and d ; (B) Cavity volume to surface ratio; (C) Cavity total surface area (D) Cavity quality factor (E) Cavity surface area (F) Wall dissipated energy regarded to the incident energy.

Further, the design was analyzed using COMSOL software to validate the performance of the TE101. Figure 5-11 (left) shows the electric field, which reaches our design purpose, creating a strong electric at the center of the cavity.

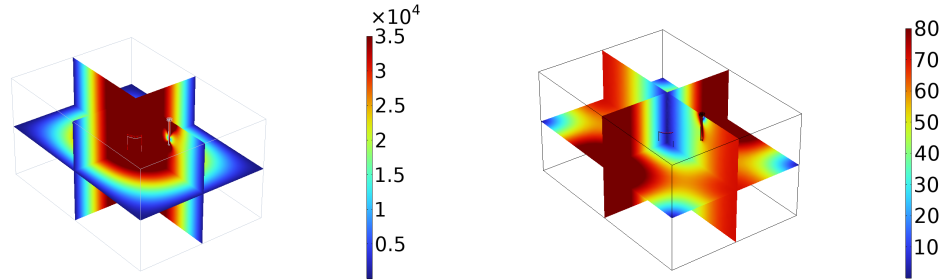


Figure 5-11. Electric field (left) and magnetic field (right) distribution at 915MHz TE101 resonant cavity

5.6.2 Development of TE102 cavity: 915MHz study case

From Chapter 5.2.2, the dimension of the cavity can be determined. When f_{102} equals 915 MHz, d should be larger than 0.367 m. Figure 5-12 (left) shows the relation between the dimensions of a and d . Figure 5-12 (right) describes the bandwidth between TE102 and TE011 when the $d = 0.367$ m was set. The larger bandwidth provides a larger operation range.

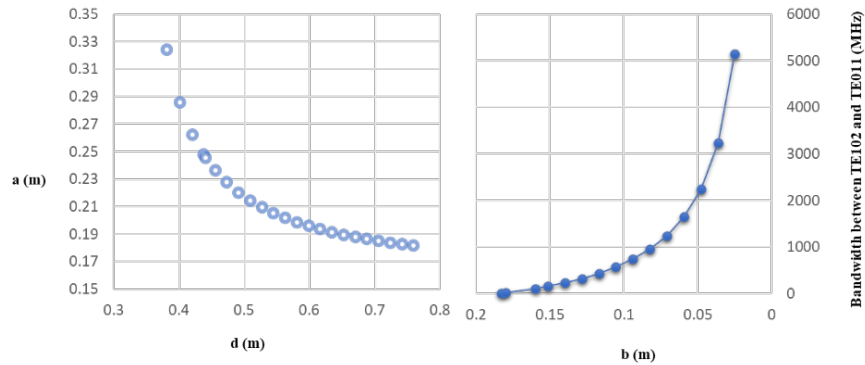


Figure 5-12. TE102 cavity dimension design. (left) Relationship between the cavity a and d ; (right) Frequency bandwidth between the dominant mode and secondary mode

Figure 5-13 (A-E) shows the simulation results for the TE102 cavity. Figure 5-13B shows the maximum magnetic field at the center of the cavity. Figure 5-13C-E shows the top view of the

magnetic field with different values of d . Figure 5-13E is selected since we are looking for a larger area at the center.

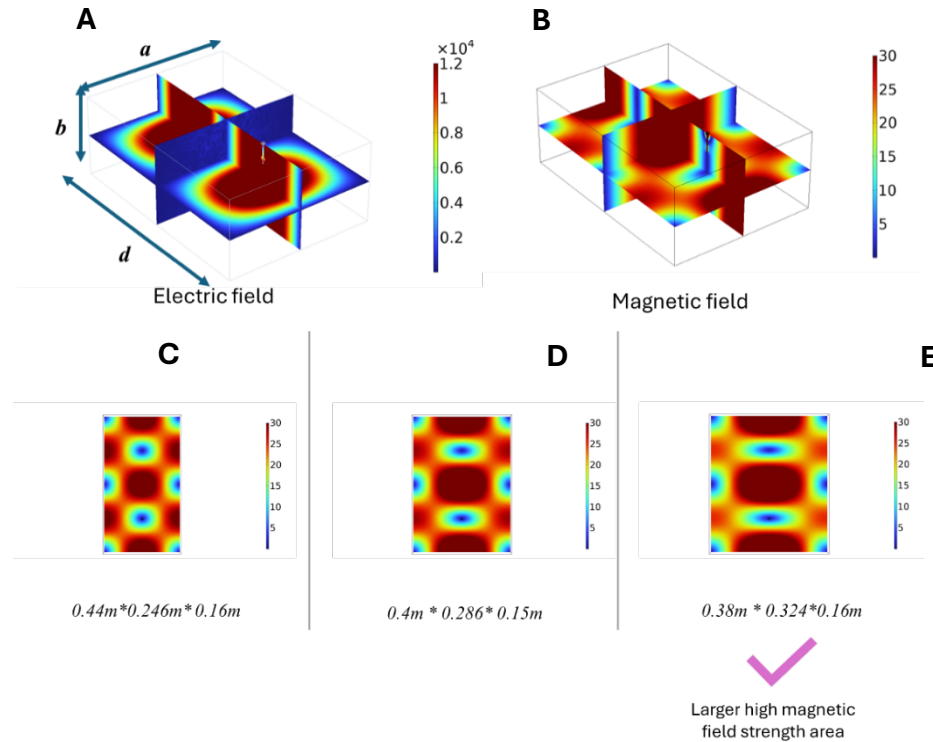


Figure 5-13. Top view magnetic field distribution of the TE₁₀ cavity at different a , b , and d values.

5.6.2 Development of TE₁₀₁ reflected cavity: 915MHz study case

From Chapter 5.2.5, the reflected cavity can be designed with a WR975 port, which has a cutoff frequency of 605 MHz based on the cutoff frequency equation in Appendix I. Figure 5-14 shows the electric field distribution with different d values. The final d is set as $d = 22$ cm to achieve the TE₁₀₁ mode and reach a similar electric field intensity (Figure 5-15) when using 3KW SSG compared to the Ren'work at 434MHz resonant cavity. Thus, the reflected cavity will also provide enough heating ability, and the system doesn't have a resonant frequency drift issue.

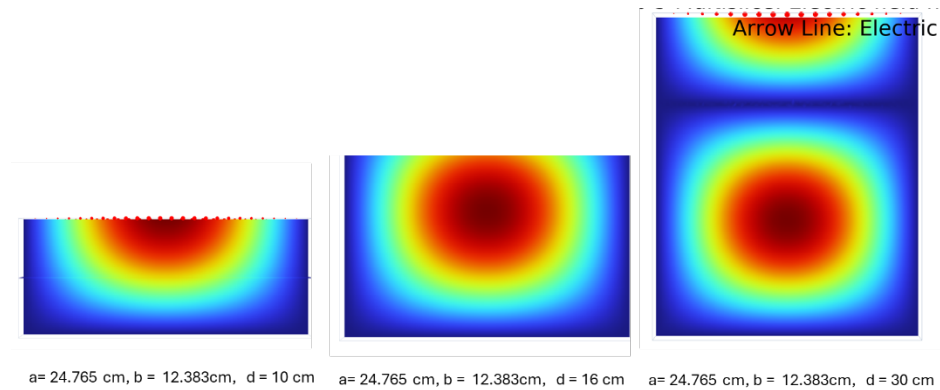


Figure 5-14. Top view of electric field distribution of the reflected cavity at different d values.

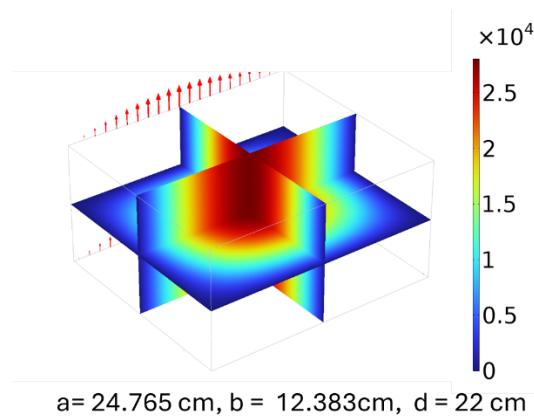


Figure 5-15. 3-D electric field distribution of the reflected cavity at designed $d = 22$ m

5.6.3 Unload cavity resonant frequency guided by material dielectric property for 910 – 930 MHz SSG

From the Eq (5-18) the unloaded cavity resonant frequency is related to the sample volume and dielectric constant. Meanwhile, the cavity volume is dependent on the unloaded cavity resonant frequency. However, the implicit equation does not always have a solution. When set $d = a$ and $b = a - 0.5$, the maximum solvable volume is 7 mL when the relative dielectric constant is 80 (room temperature water). Figure 2 shows when unloaded cavity resonant frequency you chose when you know the material type and volume. The unloaded resonant frequency increases with sample volume and dielectric constant. Designing a cavity that fits the need for a narrow bandwidth

SSG, both upper bond and lower bond should be considered. In cryopreservation, the dielectric constant increases with temperature increase. Thus, the smallest sample dielectric constant is used to make the cavity resonant frequency is at 930 MHz, and the maximum dielectric constant needs to be pre-determined to make sure the sample can be heated and pass the melting point. Currently, we don't have the data for the 915 MHz; let us imagine that the dielectric property equals 20 at the lowest temperature, and we have a sample volume of 5mL. Then, from Figure 5-16, the unloaded cavity resonant frequency should be at 953 MHz. Later we can find the dielectric constant of the material should be less than 51 to ensure it is located in between the 900 – 930 MHz range.

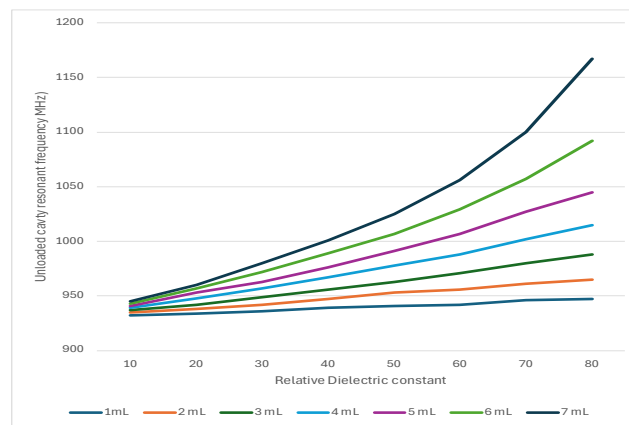


Figure 5-16. Unload cavity resonant frequency versus the sample volume and the dielectric constant

5.6.4 Determination of maximum sample volume: 434 MHz TE₁₀₁ resonant cavity with DPVP

From Luo, Pan, and Ren's work, $0.68\text{m} \times 0.4\text{m} \times 0.35\text{m}$ are selected for the cavity dimension. Thus, we can define the maximum sample volume to ensure the effectiveness of the rewarming. DPVP was selected in our research group because of its unique inverted U-shape pattern in the dielectric loss. From section Chapter 5.2.4, the maximum sample volume can be determined based on the type of vitrification solution. Q_0 can be determined by the cavity dimension shown in Appendix I. Figure 5-17 shows the maximum sample volume to its required

critical rewarming rate when the relative dielectric loss is 0.8. The higher CRR results in a smaller allowed sample volume as the input power is fixed. In addition, higher dielectric loss results in a slighter larger volume when the dielectric constant is fixed. The 915 MHz TE101 results will be performed after finishing the cavity calibration.

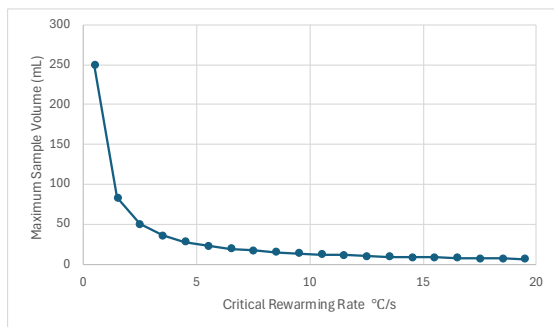


Figure 5-17. Maximum sample volume versus the critical rewarming rate (CRR)

5.7 Result of cavity simulation

5.7.1 TE101 resonant cavity

Figure 5-18 (Left) presents the temperature profile of simulation and experiment during the rewarming process of 434 MHz TE101 resonant cavity with 500 W power input. The system shows rapid rewarming and uniform temperature distribution, over 300 °C/min, for 25 mL DPVP [147]. The simulated temperature profile of the 915 MHz TE101 resonant cavity when 3 kW solid state generator will be performed after property measurement. Figure 5-18 (Right) shows the simulated temperature profile for 915 MHz with estimated dielectric properties for a 1.5 mL sample.

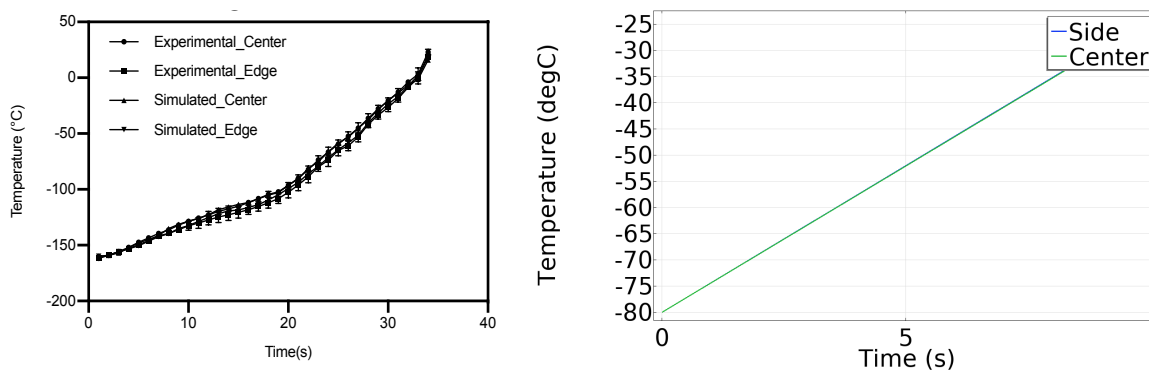


Figure 5-18. Simulated Results of 915MHz resonant cavity. (Left) Temperature profile of simulated and experimental results for 434 MHz with 25 mL DPVP solution; (Right) 1.5 mL DPVP solution temperature profile at 915 MHz

5.7.2 TE101 Reflected Cavity

The reflected cavity was simulated with 915 MHz due to the availability of SSG frequency range. Figure 5-19 shows the electric distribution on the 25 mL cylindrical sample placed at the bottom of the reflected cavity. The sample temperature increase rate is 2.2 °C/s and presents great uniformity.

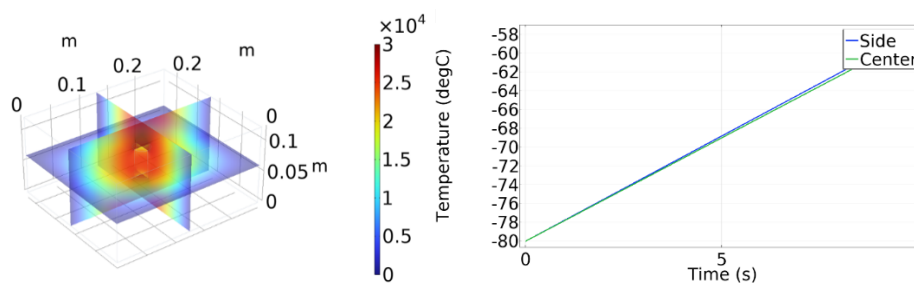


Figure 5-19. Simulated Results of 915MHz reflected cavity. (left) Electric field distribution with sample loaded into the reflected cavity; (B) temperature profile when 3 kW is used for 25 mL 50% DMSO sample.

5.7.3 TE102 rewarming

Figure 5-20A shows the material's property for potential vitrification using magnetic resonance heating. The TE102 mode cavity at 915 MHz was used to analyze the magnetic field and temperature distribution shown as Figure 5-20B-F. The results show a great temperature

uniformity and rapid rewarming at the selected three locations. The rewarming rate is about 210 °C/min which provides huge potential for further application.

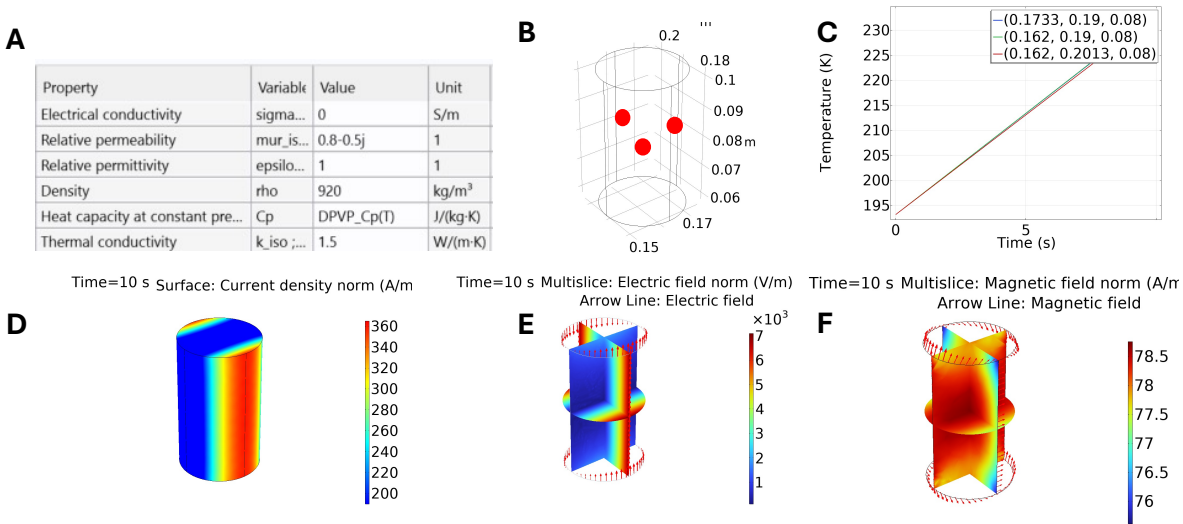


Figure 5-20. Simulation of TE102 magnetic heating. (A) A potential vitrification material property; (B) Three locations selected to study the temperature profile; (C) Temperature profile over time; (D-F) Surface current density, electric field, and magnetic field distribution

5.8 Results of Experimental Rewarming

5.8.1 Comparison of tracking and untracking resonant frequency rewarming

Figure 5-21 shows the rewarming temperature profile during the tracking and untracking resonant frequency rewarming at 100 W. The temperature profile presents similarity between -150 °C and -120 °C, which represents the resonant frequency change is ignorable and both rewarming rate is about 12 °C/min. However, when temperature beyond -120 °C, which is closer to the glass transition temperature, the rewarming rate shows significant difference. The tracking group shows average rewarming rate is 128.23 °C/min between the -120 °C and 0 °C while the untracking group shows 17.97 °C/min. The auto-resonant tracking system improves 7 times in rewarming rate by keeping the system at optimal input frequencies.

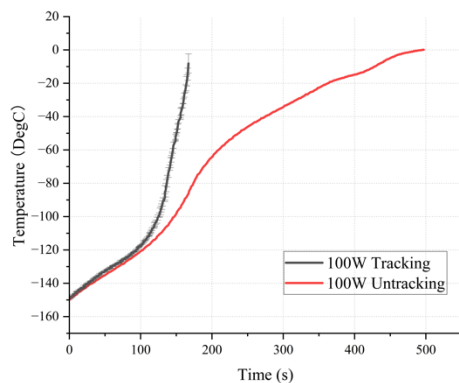


Figure 5-21. Temperature profile of tracking (n = 3) and untracking SMER system at 434MHz

5.8.2 Comparison of frozen and Vitrified rewarming

The frozen solution presents white non-transparent crystal while the vitrified sample remains clearly shown Figure 5-22. Figure 5-23 shows temperature profile of frozen and vitrified solution (30 m/m % DMSO + 30%PVP). The vitrified solution performs significantly advantage in absorbing the EM energy, which results in 375 °C/min rewarming rate. However, the frozen solution temperature increase is slow until it close to -40 °C, which is about the frozen temperature. The average rewarming rate is 107.14 °C/min. The rewarming is between -75 °C to -40 °C is 63.63 °C/min and between -40 °C to 0 °C is 266.66 °C/min. Therefore, the vitrified sample shows about 3.5 times rewarming rate on average.



Figure 5-22. Vitrified Sample (left) and frozen Sample (right)

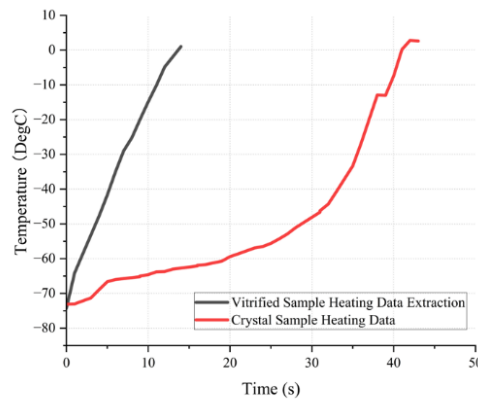


Figure 5-23. Temperature profile of vitrified and crystal sample starting from -75°C

5.9 Discussion

Avoiding re-crystallization in cryopreservation rewarming is important for the success of the biological sample's long-term preservation, especially for tissues and organs. However, the dielectric heating is governed by dielectric property and the electric field intensity. Without knowing the dielectric property of the material and the numerical derivation of wave equations in the cavities, it will be impossible to design a proper operation frequency and resonant cavity. In this study, we first tested the dielectric properties of pure and binary solutions and worked on the low temperature at 434 MHz, which is aimed at defining the best solution in our current 434 MHz cavity. Then, we Work on using either an electric or magnetic field to rapidly and uniformly rewarm the biological sample, which has been provided in both the experiment and simulation results. TE101 and TE102 were selected as two representations. TE 101 is used since it is our desired dominant mode, but the design process is also applied to TM011 or TE 011. Cylindrical resonant cavities are also popular. However, it ends up with a smaller cavity volume at the same resonant frequency in TE101 mode.

Further, the rewarming effectiveness can be achieved by some coupling factor including antenna design, adjusting the angle of the antenna, and impedance matching. The location, shape,

length, and diameter of the antenna need to be further studied in the resonant cavity to reach the optimal coupling factor. The impedance matching by applying an advanced control algorithm is also necessary to ensure the system is always at the resonant status.

Meanwhile, the auto-resonant tracking system increases the rewarming rate 7 times, which presents the significant of resonant tracking during the rewarming process. The resonant frequency tracking ensures the TE₁₀₁ mode and high electric intensity at center location of the cavity. The vitrified sample also performs high EM absorption rate. EM energy was transferred to thermal energy during the rotation of the dipolar molecular. In vitrified sample, when it is beyond the vitrified temperature, the molecule obtains freedom in rotation. At the same time, when sample temperature above the glass transition temperature and below the melting temperature. The sample is in the dangerous zone. The lower the temperature, the higher the possibility of ice nucleation but shower ice growth rath. Therefore, we need to ensure the rewarming rate is higher than the CWR to keep the freedom of rotation of molecular and avoid re-crystallization. In contrast, the frozen sample limits the molecular degree of freedom in rotation, translation and vibration since frozen is an high organized lattice, which explains the sample was heat slow under the melting temperature.

In addition, some potential methods to increase EM power absorption rate include anti-reflection coating and nanoparticles or nanotube addition. The field uniformity can be improved by applying a slab-filled resonant cavity or including two input antennas in the cavity. This allows for manipulating the maximum field intensity location and switching the E and H fields without moving the sample.

5.10 Conclusion

The current numerical study successfully provides a solution to use either electric or magnetic resonant and reflected cavities in cryopreserved rewarming. It also solves an engineering limitation of the SSG in the use of cryopreservation when the sample property changes with temperature. Then, it illustrates the relationship between the sample volume and CRR. In addition, development and optimization of the current cavity are discussed, including reducing the loss in the RF circuit, 915 MHz system prototype built, and fully automatic resonant tracking module. Later, simulation was conducted to validate the cavity design. Lastly, two set of experiments were conducted, and it shows the auto-resonant tracking system is 7 times faster than untracking SMER and the vitrified sample rewarming rate is 3.5 time faster than frozen sample from $-75\text{ }^{\circ}\text{C}$ to $0\text{ }^{\circ}\text{C}$. All of the above serves for the future development of resonant heating in cryopreservation applications and ensures the effective rewarming and viability of biological samples.

Chapter 6. STUDY OF REWARMING DYNAMICS IN 4 ML AND 20 ML VITRIFICATION SOLUTIONS FOR VARIOUS POWER INPUT

6.1 Introduction

As discussed in Chapter 5, the auto-tracking shows great advantages compared to the non-tracking heating. It's necessary to explore the auto-tracking SMER system in vitrification solution described in Table 3.1. The sample volume is determined by the cavity dimension and the signal generator operation range and available operation power. For the 915MHz, the SSG operates between 900 – 930 MHz, therefore, 4 mL is selected. 20 mL was selected for the 434 MHz based on the maximum power using automatic level control to reach steady power input. At the same time, we tracked the VSWR for the system which can help us understand the reflected power during the heating. Lastly, resonant frequency change during temperature is recorded, which can be used for frequency optimization.

6.2 Material and methods

Vitrification solution refers to Table 3.1. Solution was filled into 4mL Polypropylene cryogenic vial (Corning, AZ, US) and 20 mL plastic bottom.

Two optic fibers temperature sensor were placed in the 20mL for measuring the temperature during the cooling and rewarming process. One optimal fiber temperature sensor was placed for the 4mL cryogenic vial since the diameter is small shown in Figure 6-1.

The cooling was conducted during the LN₂ vapor for both groups in program-controlled freezer. Different cooling rates were tried to ensure successful vitrification. The rewarming of 20

mL is conducted in auto-tracking SMER (100 W (50 dbm), 250 W (54dbm), 350 W (55.5dbm) and 1000 W) and 37 °C water bath.



Figure 6-1. 4 mL and 20 mL sample holder and optic fiber temperature sensor setup

6.3 Results and Discussion

6.3.1 20mL solution experiment at 434 MHz

The 20 mL sample was cooled in 5 °C/min, 10 °C/min and 15°C/min (chamber basis) to -150 °C. Figure 6-3 shows picture taken right after open the program-controlled freezer door when the reference sample reaches -150 °C, the 5 °C/min, 10 °C/min and 15°C/min results, respectively. As shown the 30D20PVP (58kDa) can vitrified at 5 °C/min while 20D30PVP (10kDa) and 30D20PVP (10kDa) can be vitrified when cooling rate at 10 °C/min. The 10D37.5PVP (10kDa), and 40D6PVP (360kDa, Sigma) can be vitrified directly plugged into LN₂ tank, but it shows crack due to the high temperature gradient across the sample radius shown in Figure 6-3. However, 40D6PVP (360kDa, Thermofisher) can't be vitrified in all trails.

Figure 6-4 shows the five vitrified sample rewarming rate comparison between the auto-tracking SMER and water bath. The SMER system presents the around 7X faster in average and 19X faster after T_g than water bath in rewarming rate for all types of vitrified samples.

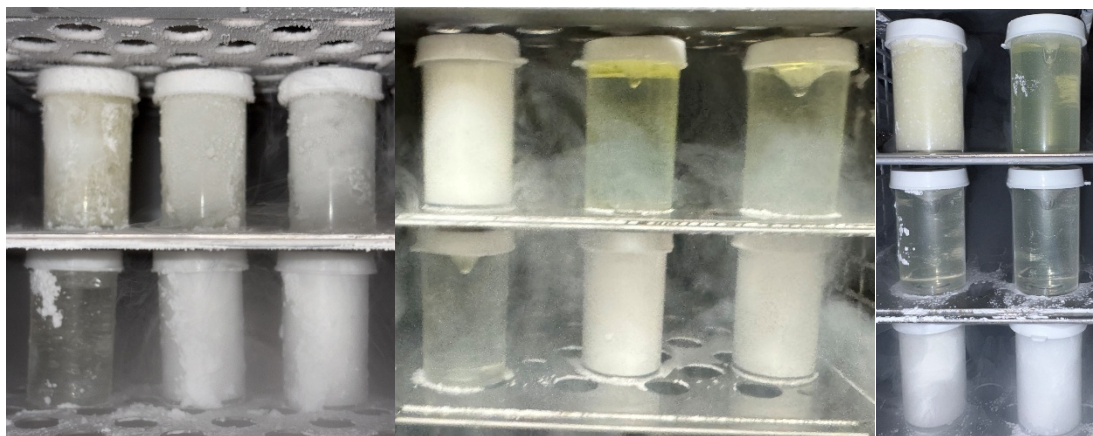


Figure 6-2 Vitrified sample after cooled to 150°C at virous cooling rates. (left) 5 °C/min, (middle) 10 °C/min, (right) 15°C/min The samples order from left-top to right follow 10D37.5PVP (10kDa), 20D30PVP (10kDa), 30D20PVP (10kDa), 30D20PVP (58kDa), 40D6PVP (360kDa, Sigma), 40D6PVP (360kDa, Thermofisher).



Figure 6-3 Vitrification solution directly Plugged into LN2 tank showing crack. (left)10D37.5PVP (10kDa), (middle) 40D6PVP (360kDa, Sigma), (Right) 40D6PVP (360kDa, Thermofisher).

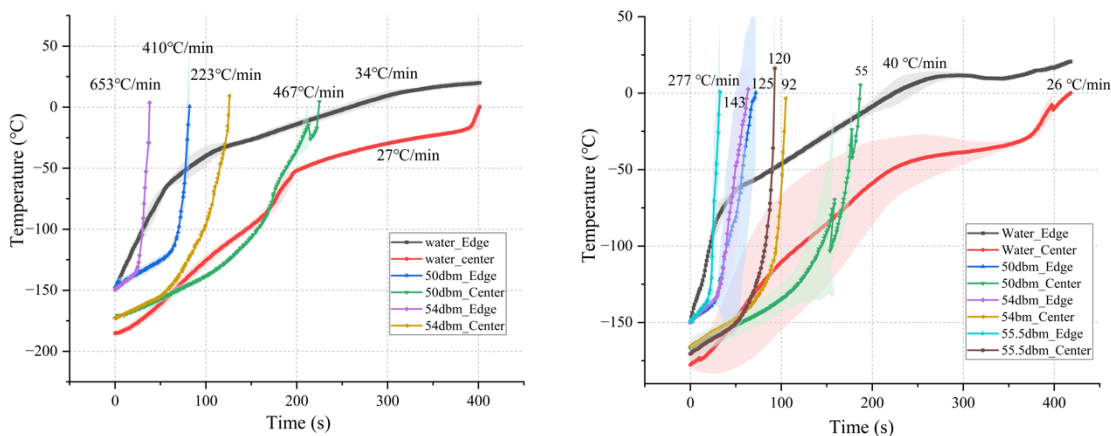


Figure 6-4 Temperature profile for water bath and auto tracking SMER. (Left) Rewarming rate of 10D30PVP(10 kDa). The rewarming rate is calculated when temperature passes the T_g . (Right) The average rewarming rate of 30D20PVP(58 kDa).

Figure 6-4 also indicates 50dbm is not sufficient for rewarming the 20 mL sample in 434MHz as the 50dbm_center (green line) initially even below the water_center (red line), which is lower than the CWR.

Figure 6-5 describes the rewarming rate of various vitrification solutions with auto-tracking SMER system using 54dbm (100W). It shows sample starts accelerated the rewarming after passing the T_g . Although the rewarming rate with auto-tracking is faster than Shen's work [48], the temperature non-uniformity is also noticed. The reason may come from the low absorption rate (low tangent loss described in Chapter 3.6) of sample when lower than T_g and the sample outer surface is hotter than the core due to the free convection. As shown in Figure 6-5, the starting temperature between edge and core has around 25 °C. This temperature gradient results in further thermal runaway in dielectric heating. In future, CPA development, this should be considered. In addition, to prevent the overheating of the outer layer, power adjustment during rewarming is also needed.

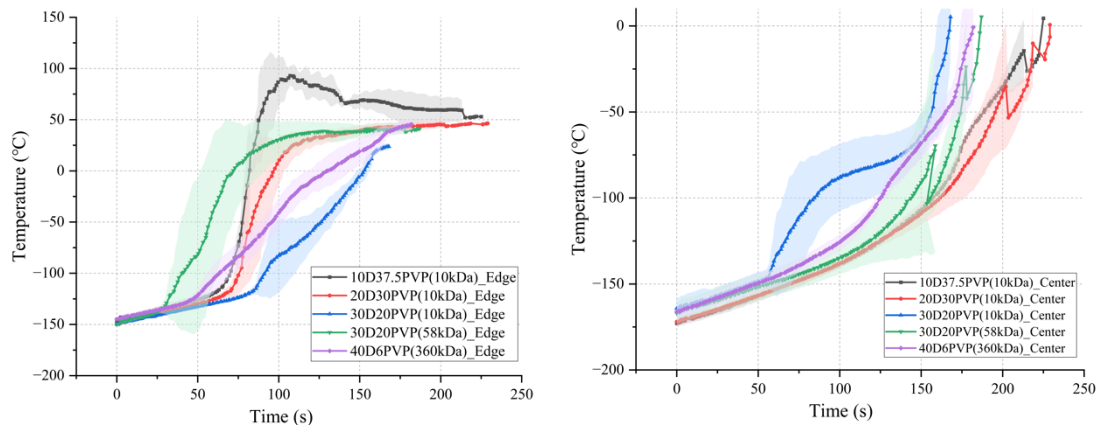


Figure 6-5 Temperature profile of edge (left) and center (right) for various vitrified sample rewarmed by auto-tracking SMER.

Figure 6-6 shows the power adjustment for 30D20PVP(58 kDa) with 50dbm (100 W), 54dbm (250 W) and 55.dbm (350 W). The higher the power input, the faster the rewarming rate

for both of the edge and core. Both 54 dbm and 55.5 dbm show similar rewarming in core area, which may avoid the recrystallization compared to 50 dbm. However, 54 dbm shows the smallest temperature gradient.

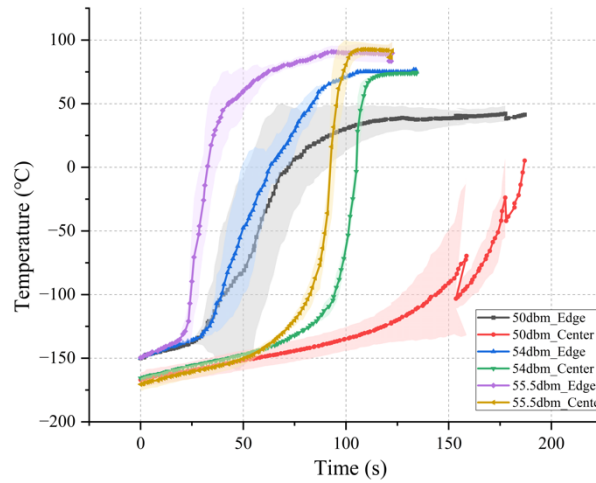


Figure 6-6 Various power input using auto-tracking SMER for 20D30PVP (58kDa)

Figure 6-7 shows the resonant frequency change, voltage standing wave ratio (VSWR), and reflects power for vitrification samples during the rewarming process. The resonant frequency is related to the dielectric constant. The measured resonant frequency decrease aligns with the dielectric constant increase with increasing temperature, which results in the resonant frequency shift described in Chapter 3.6.2. VSWR is associated with impedance matching and is commonly used in evaluating the loss of RF devices. When $VSWR = 1$, the input impedance matches the best load impedance, which can deliver the most power. Either load impedance is larger or smaller than the input impedance will result in increasing of the VSWR. Further, the reflected power can be calculated using the following equation:

$$\Gamma = \left(\frac{VSWR - 1}{VSWR + 1} \right)^2 \quad (6-1)$$

In Figure 6-7 (top-right and bottom-left), it shows the VSWR and reflected power decrease with the increasing core temperature. In addition, without the auto-tracking system, the VSWR is around 10, which results in 81% of the input power will be reflected. By using an auto-tracking system, the reflected power typically is lower than 5%, which increases energy utilized rate 1520%. Lastly, the system presents board adaptation for any time of vitrification sample and those trends can be further studied by using machine learning models to know the change of resonant frequency with the VSWR and using that to predict the sample temperature.

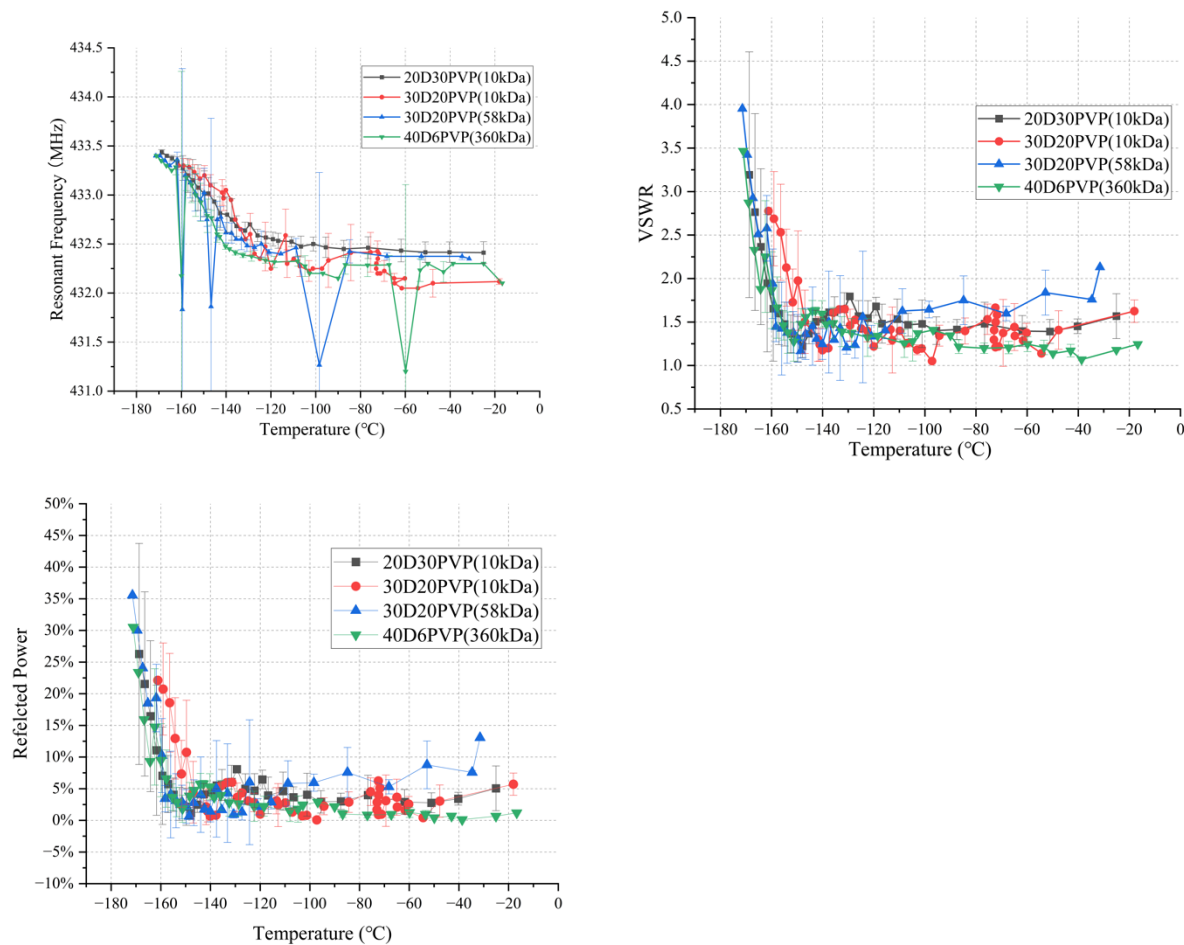


Figure 6-7 Resonant frequency change (up-left), (up-right) voltage standing wave ratio (VSWR) , and reflects power (bottom-left) for vitrification samples during the rewarming process.

6.3.2 4mL solution experiment at 915 MHz

Since the 4mL solution has a small diameter and the crack does not need to be considered. Samples were directly plugged in and fully immersed into LN₂. Figure 6-8 shows the 350 W and 1 kW temperature profile of the center location. The 1 kW is a single experiment since the power is too high for current antenna design. The 20D30PVP (10kDa) only need 15 second from -175 °C to 0 °C, 700 °C/min in average and 1250 °C/min peak. Figure 6-9 provides a guideline with about 3 power settings for rewarming 4mL in 915 MHz. The rewarming rate of 350 W, 500W and 1kW are 392 °C/min, 234 °C/min and 160 °C/min.

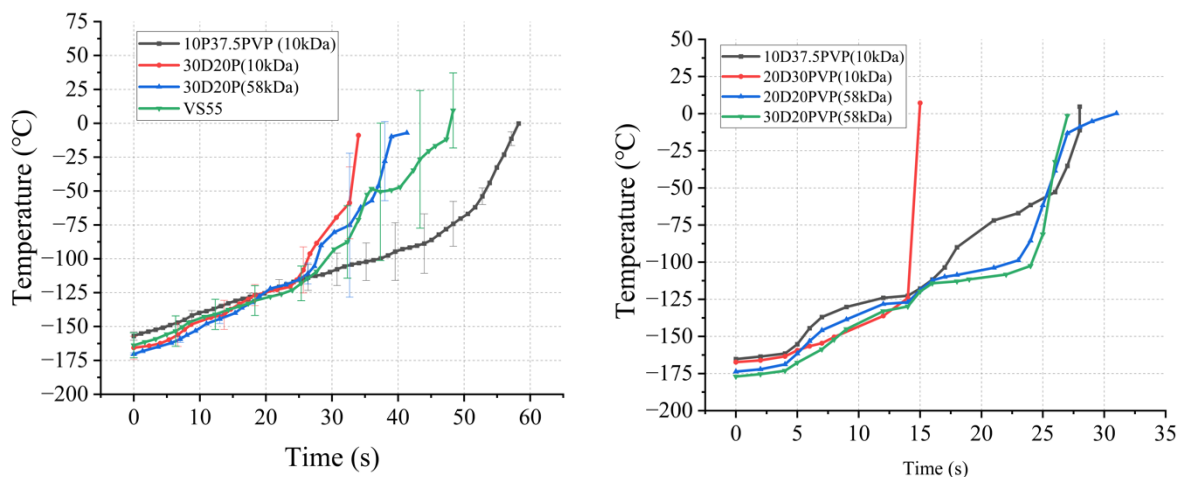


Figure 6-8. 4 mL vitrified sample rewarmed by 915 MHz SMER system with auto-tracking at 350 W (left) and 1 kW (right)

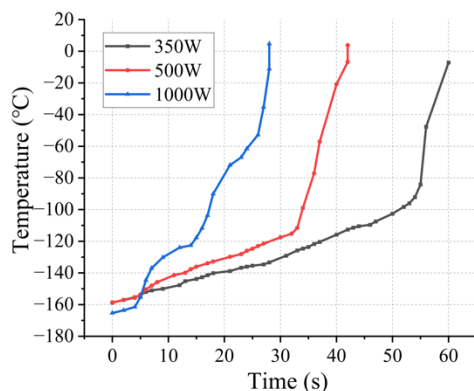


Figure 6-9 Various power for 4mL 10D37.5PVP (10 kDa) rewarming

Figure 6-10 shows five trails of resonant frequency and VSWR for the 10D37.5 PVP (10kDa). The sample shows a decreasing resonant frequency, which is similar to the 434 MHz SMER system. The resonant frequency variance becomes large after it passes the T_g , -90.58 °C since the devitrification process is meta stable. The VSWR shows the impedance matching is continuing matched by adjusting the input frequency.

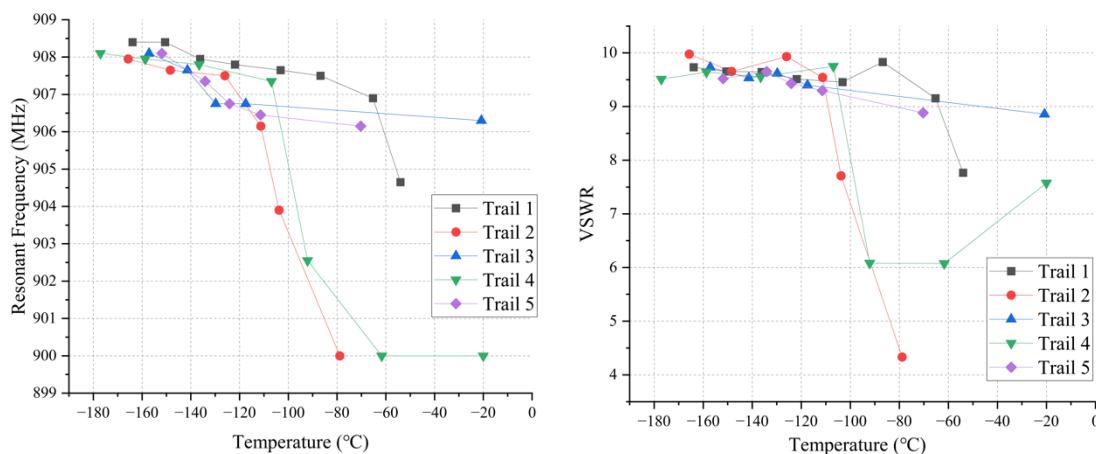


Figure 6-10. Resonant frequency and VSWR change over temperature using 10D37.5PVP (10kDa) as example.

Figure 6-11 shows the resonant frequency and reflected power, which shares the same trend with 434 MHz SMER system.

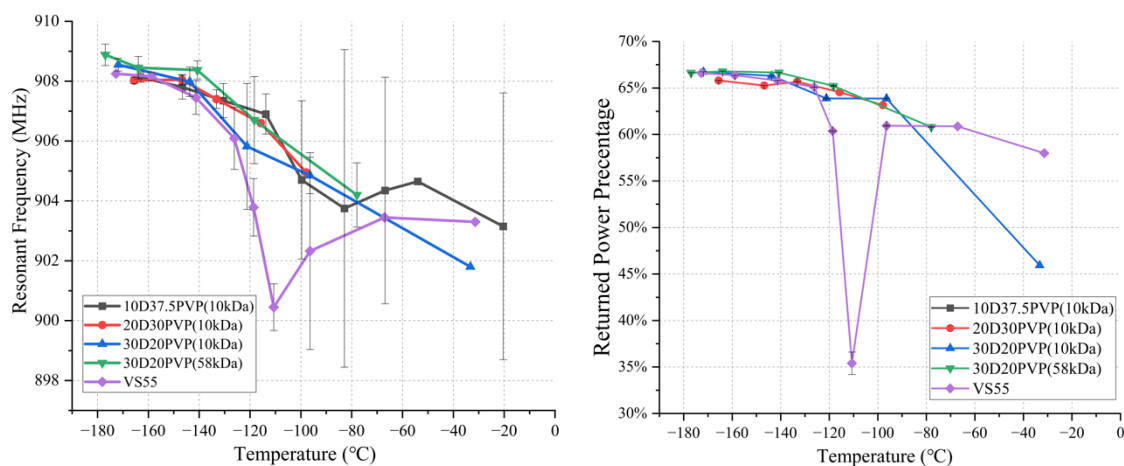


Figure 6-11. Resonant frequency and reflected power change of 915 MHz during rewarming

6.4 Conclusion

This chapter describes the use of 434 MHz and 915 MHz SMER system with auto-tracking system for 20 mL and 4 mL vitrified samples. It shows the feasibility of broadband SMER cavity described in Chapter 5. In addition, the auto-tracking system is suitable for both frequencies which can be further used in future design cavities for fitting the size or CPA need. The SMER rewarming shows more than 7 times in average or 19 times in peak of rewarming rate than water bath. Lastly, the resonant frequency and VSWR changes are monitored during the rearming process, which is not available previously. Last, by knowing the VSWR, we learn the reflected power by using the auto-tracking is less than 5% compared to the 81% non-tracking system. Therefore, the SMER systems are ready for large biological sample rewarming in either vitrified or frozen status.

Chapter 7. ELECTROMAGNETIC REWARMING OF LARGE FROZEN AND VITRIFIED JURKAT CELL SUSPENSION

7.1 Introduction

Chapter 4 discussed the cryopreservation of 4mL Jurkat cell in LN₂ with water bath rewarming. In addition, we explored the SMER system for vitrified solutions with auto-tracking in chapter 6. However, the use of SMER in large volumes of biological samples is not met yet. In this chapter, 10 mL and 20 mL Jurkat cell suspension cryopreserved in -80 °C and -196 °C are studied. 10 mL sample was cooled to -80 °C and store in -80 °C freezer and 20 mL sample was cooled to -196 °C and stored in LN₂ tank. Multiple rewarming methods was conducted in -80 °C group. SMER system and water bath were used to warm sample from -196 °C.

7.2 Material and methods

7.2.1 10 mL cell suspension experiment

The same type of Jurkat cell was cultured and used as described in Chapter 4.2.1. The Jurkat cells were cultured, and the live cell count (n =4) is at $9.71 \times 10^5 \text{ mL}^{-1}$ before all treatments. Then aliquoted 5 mL to 10 mL plastic tube for later use (n = 9).

20% (m/m) DMSO stock solution was prepared on the experiment day and placed on ice before introducing to cell suspension. Later, the stock solution was added to cell suspension dropwise until it reach 10 mL. Then, samples are transferred to -80 °C.

7.2.2 20 mL cell suspension experiment

The same type of Jurkat cell was cultured and used as described in Chapter 4.2.1. The Jurkat cells were cultured, and the live cell count (n =12) is at $1.12 \times 10^6 \text{ mL}^{-1}$ before all

treatments. Then aliquoted 5 mL to 20 mL plastic tube for later use ($n = 6$). 2step_CPA loading method was applied. First, 1:1 mix the cell suspension with 26.6% (m/m) DMSO and place it on ice for 3min. Then, Later, draw 5 ml cell suspension and add 15ml 9% (m/m) DMSO and 0.50 % (m/m) Polyvinylpyrrolidone (PVP)) until it reaches 20 mL. After loading the PVP, shake the solution gently to uniform the mixture and place on ice for 3 min before transferring to the LN₂ tank. The final concentration reaches 10% (m/m) DMSO and 37.5% (m/m) PVP in CPA and Jurkat cell suspension mixture.

The viability and live-cell concentration were determined by the Trypan Blue solution (ThermalFisher, Waltham, MA).

7.2.3 Rewarming methods

10 mL experiments are divided into three groups classified by different rewarming methods (three each group, $n = 3$) including 434 MHz SMER, water bath and air.

10 mL experiments are divided into two groups classified by different rewarming methods (three each, $n = 3$) including 434 MHz SMER, and water bath.

The water bath is in a non-shaking condition. Sample were rewarmed until thermal couple reading reached 0 °C, and no obvious ice crystal was observed in all methods.

7.2.4 Assessment methods

The recovery and proliferation rate assessment follow the same process, which refers to Chapter 4.2.6 and Chapter 4.2.7.

7.3 Results and discussion

7.3.1 Determination of the recovery rate of the Jurkat cells

The membrane integrity of the Jurkat cells with different CPA composition was tested by Typan Blue staining solution. As shown in Figure 5-1 (top), the recovery rate of 10 mL Jurkat cells cryopreservation of SMER, waterbath, and air were $98.17\% \pm 7.12\%$, $94.00\% \pm 6.56\%$, and $58.56\% \pm 17.00\%$, respectively. The mean values $\pm$ standard deviations were determined based on twelve replicates ($n=12$). Figure 5-1 (bottom) shows the recovery rate of 20 mL Jurkat cells cryopreservation of SMER, and waterbath were $32.68\% \pm 2.52\%$, $72.16\% \pm 6.22\%$, respectively.

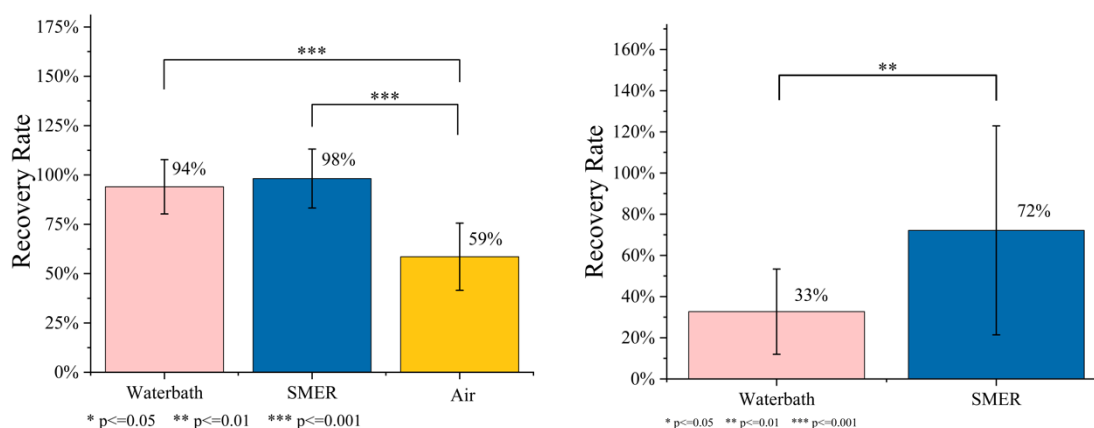


Figure 5-1. The recovery rate of 10 mL (Top) and 20 mL (bottom) experiment

When 10% DMSO is used for 10 mL jurkat cell suspnsion and stored in -80°C , both water bath and EM shows great recovery rate. However, the EM takes 20s in rearming it while the wather bath cost 3 mins. Moreover, directly inmmersing into water may cause contaminatin of future cellular thearpy applications. In addition, air rewarming was tested as well. The recover rate is much lower compared to the water bath and SMER system.

When 2step_DPVP is used to vitrify 20 mL jurkat cell suspension in LN_2 environment. The SMER perform supplier than the waterbath as the waterbath rewarming results the recrystalization

and non-uniformity. Thus, SMER has advantage in large volume sample rewarming and bring the future exploration in tissue and organ cryopreservation.

7.3.2 Determination of the metabolic activity of the post-thawed cells

The post-thawed Jurkat cells were incubated at 37 °C with 5% carbon dioxide, the metabolic activity (n = 16) was assessed by alamarBlue assay after 18hr. The operation procedures and data analysis follow Chapter 4.3.2. Figure 5-2 shows the metabolism or proliferation rate after rewarming. The mean values $\pm$ standard deviations were determined based on twenty-four replicates (n = 24).

After 18 hours incubation, the waterbath and the SMER show similar proliferation, which means both EM and waterbath are sufficiently in protecting the functionality when sample size is small. However, when sample size increases to 20 mL and the storage temperature is lowered to -196 °C with vitrification solution, the EM shows great performance as it has faster rewarming rate discussed in Chapter 6.

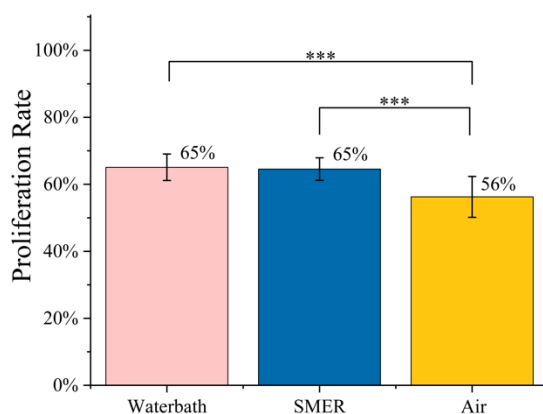


Figure 5-2. Determination of the post-thawed cells metabolic activity by alamarBlue

7.4 Conclusion

In this chapter, 2 sets of experiments using Jurkat cell were conducted. For 10 mL, sample was used 10% DMSO and cooled to -80 °C. Both recovery and proliferation rate are high. However, when sample size further increases to 20 mL and using verification solution described in Chapter 4. The SMER system has significantly high recovery and proliferation rate compared to the waterbath, which illustrates the high rewarming rate avoiding the recrystallization. Thus, SMER rewarming has its advantage in rewarming both frozen and vitrified samples.

Chapter 8. Summary and future work

8.1 Summary

Cryopreservation is furnishing new research areas including the development of the CPAs, prototyping the related device, understanding the mechanism of cryoinjury, exploring the possibility in volumetric heating, and most importantly, scaling up in both the size and the complexity of biological samples. Unfortunately, the cryopreservation of large volume of biological samples is still limited by the cooling and rewarming. Vitrification usually requires high concentration of the permeating CPA, which increases osmotic injury and the toxicity. At the same time, rewarming methods are still under development for achieving uniform volumetric heating.

In order to lower the DMSO usage in vitrification, high concentration of PVP polymer was applied and analyzed. The PVP shows good biocompatibility with Jurkat cells and the proposed solutions in Table 3-1 are able to vitrify in 25 mL. Lager 4mL Jurkat cells was added the vitrification solution with 1 step and 2 step to understand the diffusion rate in vitrification solution. The samples were cooled by directly plugged into the LN₂ tank and rewarmed with conventional 37 °C waterbath after 2 days storage. The results shows that 2step_DPVP solution is superior in both recovery rate and proliferation, which tells the protection of DPVP during the cooling by forming verification status. In addition, 2step is better than 1step means the DMSO and water diffusion rate is slowed by the high viscosity.

Although we achieved successful results in rewarming 4mL vitrified sample by water bath. The recrystallization is still observed at the core. Traditional cryopreservation techniques are largely limited to small and simple biological systems, primarily due to the absence of efficient rewarming methods. For larger specimens—such as tissues or organs—conventional approaches like water bath immersion or passive air convection are inadequate. These methods typically result

in slow warming rates that promote ice recrystallization, leading to cellular damage or rupture. Additionally, the formation of steep temperature gradients within the sample can induce internal thermal stress, increasing the risk of structural fractures.

Therefore, a volumetric heating method is desired. The dielectric heating is a promising solution. SMER cavity can provide a high focused electric field at the designated location based on the mode selection. We systemically analyze the design of SMER in broadband frequency and use two frequencies (434 MHz and 915 MHz) as examples. However, the resonant frequency of the SMER system keeps changing with the change in the material dielectric properties, so-called drift or off-resonance. When the system is off-resonant, the impedance matching becomes worse, and most of the input power will be reflected, so the sample will not be heated rapidly. We have developed a fully automated resonant tracking system with using 2 RF switch. The system tracks the resonant frequency every 5 seconds. We further validated the auto-tracking system is about 4X better than non-tracking SMER system in 100W. The higher the power usage, the better the performance, since the non-tracking always reflected most of the power. In addition, we found the vitrified sample is easier to rewarm compared to frozen samples in SMER, which may be due to the limitation of the molecular movement during the lattice arrangement.

Later, we test the 4mL and 20mL in various input power of SMER system. With increasing power, the rewarming rate is significantly increased, but the sample temperature performs non-uniformity when sample radius increases. Additionally, we found although the SSG has an advantage in power level, the adjustable range limited the sample volume can be heated in 915 MHz. The EM system also indicates the sample will increase the ability in absorbing the electric field energy when passing the glass transition temperature. Thus, the CPA glass transition temperature is important not only the cooling process but also the rewarming.

Then SMER system was applied to rewarming 10mL from -80 °C with 10% DMSO and 20 mL from LN₂ with 2step_DPVP CPA loading method. For the 10 mL group, wasterbath and EM shows comparable results in both recovery rate and proliferation while the water bath needs 3 min when fully immersed while the EM need around 20 second at 350W input. Later, the 20 mL vitrified sample from LN₂ tank is rewarmed by water bath and SMER. The SMER shows significantly higher recovery rate and proliferation compared to water bath, which proves the water bath is not sufficient in rewarming large, vitrified samples. Lastly, the SMER system was applied to the mice kidney tissues and tested based on alamarBlue and LDH assay for its metabolism activity. The SMER system shows better results in both conventional vitrification CPA and DPVP.

8.2 Future work

The promising outcomes from both large-volume cell and tissue experiments highlight the capability of the electromagnetic resonance rewarming system to achieve rapid and uniform heating. These results represent a significant step forward and offer strong potential for addressing the longstanding challenges in organ preservation. In order to being successful in organ preservation, we need to work on CPA development, CPA perfusion, Cooling protocol optimization, SMER system optimization and organ functionality assessment.

CPA development is related to biocompatibility and toxicity. More importantly, it determined the CCR and CRR. A multi-component of vitrification solution should be developed, which targets to lower the DMSO, and reduce the viscosity in 0 °C to room temperature for better handling. The future application may involve the PVP, PVA and trehalose mixture by further exploring the Physics of Condensed Matter. In addition. as the electric field is selected as our rewarming source, the CPA dielectric property is important to assist the rewarming process. Thus,

high tangent loss material should be designed by molecular dynamics and molecular relaxation model.

Then the perfusion system should be developed for long periods of organ CPA loading. The system should gradually increase concentration to minimize the osmotic injury. Further, the system is looking to have self-adjusting function based on the viscosity change and flow rate change with PID control or physical enhanced AI model. The mice liver vitrification was successful by immersing the liver in vitrification solution in a week shown in Figure 7-1. More study in perfusion devices is needed.



Figure 8-1. Mice liver vitrification. Left is before cooling and right is vitrified.

Cooling process is coupled with CPA design and screening, which needs to study the glass transition temperature and heat capacity. Then, an optimized cooling protocol should be developed based on the program-controlled freezer. In addition, an electric field assisted cooling method may be explored. The water molecular has 3 degrees of freedom and vitrification solution has 2 (vibration and rotation) and crystal ice only contain 1 (vibration). Thus, the ice formation may be suppressed with the use of the alternative electric field during the cooling process. The nanoparticles including nanocarbon, nanopolymer, nanometal, nonoceramic, ice inhibitor, and anti-frozen protein should be considered in CPA development.

Although the SMER performed well in large volume cell and tissue rewarming, further optimization of SMER system is needed. First, the SMER system should be prepared to large volume animal or human organs, which might lower the input frequency and gain larger cavity dimension. Secondly, a high-power amplifier is needed for samples over 50mL. Thirdly, the energy input method should be further investigated. Currently, the input energy transmits using coaxial cable and the electric field is excited by probe antenna. At the current setup, the coupling between the probe antenna is very curial. The couple affect the VSWR, which determines the reflected power. Thus, the location and the length of the antenna should be optimized to reach the lowest VSWR and highest the Q factor. However, the coaxial cable and the probe antenna are not suitable for power over 1 kW. Thus, when power leveled up, the reflected cavity might be a possible method. However, the reflected cavity is only double the energy input. The proper method to transmit the power into the resonant cavity still needs to be explored. In addition, the whole RF circuit should be studied including the source, cables, each RF component, antenna and sample.

Fourth, monitoring system and moisture control system should be equipped with new cavities as the biological sample mostly doesn't contain the temperature sensor. Multiple infrared detectors should be monitoring the outer surface and the bottom. Moisture control should apply nitrogen gas to avoid condensation on the sample holder surface and form ice to reduce the EM absorbing rate. AI tuning by studying the frequency change can be used to make a frequency cut-off-based algorithm.

Fifthly, although RF switch tracking shows promising result in improving the rewarming rate, it requires 1.7 second pulsing time for RF switch back and forth (0.2s) and the measurement of the VNA (1.5s). A VNW at a faster scanning rate can reduce the pulsing significantly. Furthermore, a model free control algorithm such as extreme seeking control, reinforcement

learning based control, stochastic perturbation optimization and Bayesian optimization, shall be used to explore the possibility to avoid the pulse. In mechanical and electrical aspects, an automatic stub tuning system may be an alternative way, but the cost is high at current market price.

Last and perhaps the most important, the thermal runaway needs to be considered when large samples are involved. Thermal runaway is curial when enlarging the sample as the penetration depth and the electric field. Starting with the simulation of the various sample size and electric field uniformity, the temperature profile should be studied by connecting the CPA screening results. Then, the electric field uniformity can be further enhanced by introducing slab, so called slab loaded resonant cavities. By using that, the previous normal distribution of electric field will be flat at the center location, which benefits the electric field uniformity across the sample. An additional method for increasing the absorbing is about the bottom design. The dielectric property difference between the sample and air results in the reflected wave and lower the absorption of EM energy. By using an anti-reflection layer, similar to stealth coating technology in fighter. We can confine the EM energy not being reflected when it right after passing the glass transition temperature to increase the rewarming rate and reduce the possibility of recrystallization. Another method is relying on the phase adjust between input. The peak location of the electric field can be adjusted by two inputs at different phase. When introducing two input probes, it can heat the low temperature regain and reduce the rewarming rate for high temperature. Lastly, a small shaking platform may build the inside of the cavity and vibrate the sample during the heating to a uniform temperature.

Appendix I

Wave Equation of Parallel Waveguide

Dimension	E Field	H Field
x	$\frac{j\beta}{k_c} B \sin\left(\frac{n\pi}{b} y\right) e^{-j\beta z}$	0
y	0	$\frac{j\beta}{k_c} B \sin\left(\frac{n\pi}{b} y\right) e^{-j\beta z}$
z	0	$B \cos\left(\frac{n\pi}{b} y\right) e^{-j\beta z}$

Wave Equation of Rectangular Waveguide

	E Field	H Field
x	$\frac{j\omega\mu n\pi}{k_c^2 b} AC \cos\left(\frac{m\pi}{a} x\right) \sin\left(\frac{n\pi}{b} y\right)$	$\frac{j\beta n\pi}{k_c^2 b} AC \cos\left(\frac{m\pi}{a} x\right) \sin\left(\frac{n\pi}{b} y\right)$
y	$-\frac{j\omega\mu m\pi}{k_c^2 ba} AC \sin\left(\frac{m\pi}{a} x\right) \cos\left(\frac{n\pi}{b} y\right)$	$\frac{j\beta m\pi}{k_c^2 a} AC \sin\left(\frac{m\pi}{a} x\right) \cos\left(\frac{n\pi}{b} y\right)$
z	0	$AC \cos\left(\frac{m\pi}{a} x\right) \cos\left(\frac{n\pi}{b} y\right)$

Wave Equation of Resonant Cavity

	E Field	H Field
x	0	$\frac{j2ACk_z k_x \sin(k_x x) \cos(k_z z)}{k^2 - k_z^2}$
y	$\frac{-2AC\omega\mu k_x \sin(k_x x) \sin(k_z z)}{k^2 - k_z^2}$	0
z	0	$-j2AC \cos(k_x x) \sin(k_z z)$

Cut off frequency of TE mode $f_c = \frac{1}{2\sqrt{\epsilon\mu}} \sqrt{\left(\frac{m}{a}\right)^2 + \left(\frac{n}{b}\right)^2}$

Unloaded Quality Factor

$$Q_0 = \frac{\pi \mu f_{101} a b}{R_s d} \frac{\left(\frac{a^2}{d^2} + 1\right)}{\left[\left(\frac{a}{b}\right)\left(\frac{a^2}{d^2} + 1\right) + \left(\frac{2b}{d}\right)\left(\frac{a^3}{d^3} + 1\right)\right]} = \frac{\pi^2 \eta b}{2 R_s d} \frac{\left(\frac{a^2}{d^2} + 1\right)^{3/2}}{\left[\left(\frac{a}{b}\right)\left(\frac{a^2}{d^2} + 1\right) + \left(\frac{2b}{d}\right)\left(\frac{a^3}{d^3} + 1\right)\right]}$$

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