NANOWARMING AND FORMULATION OF STABLE MAGNETIC VITRIFICATION SOLUTIONS

By

EMAN SHRETEH

A THESIS PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2018
To my family,
Maher and Rola Shretreh
Samia, Hany, Reem, Saleh and Raghad
ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Carlos Rinaldi for giving me the opportunity to build the basis for nanowarming project and guiding me throughout my thesis work. Thank you for giving me the opportunity to work independently and allowing me to explore the various aspects of this project as I learned tremendously about a field that I had very little knowledge in when I first started.

I am also thankful to Ana Carolina Bohorquez and Mythreyi Unni from the Rinaldi lab for their assistance in answering questions I had with regards magnetic nanoparticles and Igor software, and I would also like to thank all members of the Rinaldi lab for their support.

Next, I would like to thank my parents, and my siblings for always supporting and encouraging me. I am also thankful for my friends for their continuous support.

Last, I would to thank the J. Crayton Pruitt Family Department of Biomedical Engineering and the University of Florida for providing the infrastructure for this research.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>4</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>7</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>8</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>10</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>11</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1 INTRODUCTION</td>
<td>13</td>
</tr>
<tr>
<td>1.1 Current Challenges and Motivation</td>
<td>13</td>
</tr>
<tr>
<td>1.2 Objectives</td>
<td>14</td>
</tr>
<tr>
<td>2 BACKGROUND</td>
<td>15</td>
</tr>
<tr>
<td>2.1 Significance</td>
<td>15</td>
</tr>
<tr>
<td>2.2 Methods of Tissue and Organ Preservation</td>
<td>16</td>
</tr>
<tr>
<td>2.3 Cryopreservation by Vitrification</td>
<td>18</td>
</tr>
<tr>
<td>2.3.1 Background</td>
<td>18</td>
</tr>
<tr>
<td>2.3.2 Basis of vitrification</td>
<td>19</td>
</tr>
<tr>
<td>2.3.3 Cryoprotectant Solutions (CPAs)</td>
<td>20</td>
</tr>
<tr>
<td>2.3.4 Applications: Cells, Tissues and Organs</td>
<td>22</td>
</tr>
<tr>
<td>2.3.5 Challenges</td>
<td>25</td>
</tr>
<tr>
<td>2.3 Nanowarming Phenomena</td>
<td>30</td>
</tr>
<tr>
<td>2.4.1 Current Rewarming Modalities</td>
<td>30</td>
</tr>
<tr>
<td>2.4.2 IO MNP</td>
<td>31</td>
</tr>
<tr>
<td>2.4.3 Relevant Nanowarming Studies</td>
<td>32</td>
</tr>
<tr>
<td>3 MATERIALS AND EXPERIMENTAL METHODS</td>
<td>34</td>
</tr>
<tr>
<td>3.1 Materials</td>
<td>34</td>
</tr>
<tr>
<td>3.2 Methods</td>
<td>35</td>
</tr>
<tr>
<td>3.2.1 Characterization</td>
<td>35</td>
</tr>
<tr>
<td>3.2.1.1 Dynamic Light Scattering</td>
<td>35</td>
</tr>
<tr>
<td>3.2.1.2 Rheology</td>
<td>37</td>
</tr>
<tr>
<td>3.2.1.3 SQUID Magnetometer</td>
<td>38</td>
</tr>
<tr>
<td>3.2.1.4 Thermal Energy Dissipation</td>
<td>38</td>
</tr>
<tr>
<td>3.2.1.5 Temperature Sensors</td>
<td>39</td>
</tr>
<tr>
<td>3.2.2 mCPA Formulation</td>
<td>41</td>
</tr>
<tr>
<td>3.4.2 Colloidal Stability</td>
<td>42</td>
</tr>
<tr>
<td>Table</td>
<td>Description</td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
</tr>
<tr>
<td>3-1</td>
<td>Concentration and composition of CPA solutions</td>
</tr>
<tr>
<td>3-2</td>
<td>Temperature ranges of various temperature sensors</td>
</tr>
<tr>
<td>4-1</td>
<td>A summary of viscosity values used in DLS measurements</td>
</tr>
<tr>
<td>4-2</td>
<td>Mean hydrodynamic diameter of particles suspended in CPAs and obtained by DLS over two weeks period</td>
</tr>
<tr>
<td>4-3</td>
<td>Mean hydrodynamic diameter of particles in CPAs obtained by DLS pre-vitrification and post nanowarming</td>
</tr>
<tr>
<td>4-4</td>
<td>Initial heating rates with and without particles and with and without AMF</td>
</tr>
<tr>
<td>4-5</td>
<td>Initial rewarming rates and SAR values of mVS55 upon exposure to various AMF amplitudes</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Organ shortage- gap between supply and demand [5]</td>
<td>16</td>
</tr>
<tr>
<td>2-2</td>
<td>Path followed temperature vs concentration plot of propane-1,2- diol/ water phase diagram. T_h is the homogenous nucleation temperature, T_g is the transition glass curve, T_m is the melting point curve, and T_d is the devitrification [12].</td>
<td>20</td>
</tr>
<tr>
<td>2-3</td>
<td>Critical cooling and rewarming rates of various concentration of ethylene glycol in water [48, 50].</td>
<td>27</td>
</tr>
<tr>
<td>2-4</td>
<td>Rewarming of vitrified tissues using nanowarming for bulk multicellular tissues provides rapid and uniform heating, which ameliorates devitrification and fracture [71].</td>
<td>31</td>
</tr>
<tr>
<td>2-5</td>
<td>Mechanisms of magnetic nanoparticles relaxations upon exposure to AMF [68].</td>
<td>32</td>
</tr>
<tr>
<td>3-1</td>
<td>Schematic Cup and Bob geometry [40].</td>
<td>37</td>
</tr>
<tr>
<td>3-2</td>
<td>Tips of temperature sensor probes. (A) Fiber optics. (B) cryogenic fiber optics. (C) thermocouple type T.</td>
<td>39</td>
</tr>
<tr>
<td>3-3</td>
<td>Schematic of FO sensor [67].</td>
<td>41</td>
</tr>
<tr>
<td>3-4</td>
<td>Formulated 1 ml of three mCPAs and PBS 1X of particles suspension.</td>
<td>42</td>
</tr>
<tr>
<td>3-5</td>
<td>An overview of pre-vitrification and post nanowarming stability study. (Left) mVS55 during IH. (Top) vitrified mVS55 as sample was taken from LN2. (Bottom) mVS55 post nanowarming.</td>
<td>43</td>
</tr>
<tr>
<td>3-6</td>
<td>IO MNP samples.</td>
<td>44</td>
</tr>
<tr>
<td>3-7</td>
<td>Modified TC_T including normal, taped soldered and soldered.</td>
<td>45</td>
</tr>
<tr>
<td>4-1</td>
<td>Magnetization curve of PEG- 2000 coated iron oxide prior to adding to CPAs using SQUID.</td>
<td>47</td>
</tr>
<tr>
<td>4-2</td>
<td>Size characterization of PEG- 2000 coated iron oxide suspended in DIW prior to adding to CPAs using DLS.</td>
<td>48</td>
</tr>
<tr>
<td>4-3</td>
<td>Rheology measurements of CPA formulations characterizing viscosity of CPAs solutions using Rheometer at two cycles of increasing and decreasing shear rates.</td>
<td>49</td>
</tr>
<tr>
<td>4-4</td>
<td>Stability pf PEG- 200 coated IO MNP suspended in CPAs was assessed using DLS at different time points over two weeks showing no aggregation and colloidal stability in all solution except in DGPE.</td>
<td>51</td>
</tr>
</tbody>
</table>
Pre-vitrification and post rewarming size characterization of particles assessed using DLS ...............................................................52

Temperature vs time of three temperature sensors: TC_T, FO and cryo FO upon exposure to AMF. (A) IO MNP samples. (B) DIW samples..........................................................54

Temperature vs Time plot of DIW upon exposure to AMF. (A) plot with all temperature sensors. (B) plot without taped soldered..........................................................55

Temperature vs Time plot of Three temperature sensors: TC_T, TC_E and FO in DIW sample. ..................................................................................................................................................................56

Temperature vs Time plot of TC_E in DIW upon exposure to AMF...............................................................57

Temperature vs Time plot of TC_kit and TC_T1 pairing when subjected to AMF........58

Temperature vs Time plot of TC_T1 and TC_T2 pairing when subjected to AMF........59

Temperature vs Time plot of TC_kit and TC_E pairing when subjected to AMF........59

Temperature vs Time plot of TC_E and TC_T1 pairing when subjected to AMF........59

Temperature vs Time plot of TC_kit1 and TC_kit2 pairing when subjected to AMF ......60

(A) Full temperature profile including cooling and rewarming of mVS55 in an AMF and VS55 at RT. (B) Nanowarming of mVS55 in an AMF (37.5 kA/m and 348 kHz) compared to mVS55 at RT with no field (No AMF) and VS55 without IO MNP..............62

Control of heating rates of mVS55 through varying AMF amplitudes .........................63

Pre-vitrification and post rewarming size characterization of PEG-5000 coated IO MNP assessed using DLS ..............................................................................................................64
<table>
<thead>
<tr>
<th>ABBREVIATION</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFP</td>
<td>Antifreeze proteins</td>
</tr>
<tr>
<td>AMF</td>
<td>Alternating magnetic field</td>
</tr>
<tr>
<td>CPA</td>
<td>Cryoprotectant agents</td>
</tr>
<tr>
<td>Cryo FO</td>
<td>Cryogenic fiber optic</td>
</tr>
<tr>
<td>DIW</td>
<td>Deionized water</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>FO</td>
<td>Fiber optic</td>
</tr>
<tr>
<td>IH</td>
<td>Induction heating</td>
</tr>
<tr>
<td>IO MNP</td>
<td>Iron oxide magnetic nanoparticles</td>
</tr>
<tr>
<td>mCPA</td>
<td>Magnetic cryoprotectant agents</td>
</tr>
<tr>
<td>mVS</td>
<td>Magnetic vitrification solutions</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>SAR</td>
<td>Specific absorption ate</td>
</tr>
<tr>
<td>SQUID</td>
<td>Superconducting Quantum Interference Device</td>
</tr>
<tr>
<td>TC</td>
<td>Thermocouple</td>
</tr>
<tr>
<td>TC_E</td>
<td>Thermocouple type E</td>
</tr>
<tr>
<td>TC_T</td>
<td>Thermocouple type T</td>
</tr>
<tr>
<td>TC_T_i</td>
<td>Thermocouple type T; i = 1, 2</td>
</tr>
<tr>
<td>THP</td>
<td>Thermal hysteresis</td>
</tr>
<tr>
<td>TMS</td>
<td>Thermo-mechanical stress</td>
</tr>
<tr>
<td>VS</td>
<td>Vitrification solutions</td>
</tr>
</tbody>
</table>
Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

NANOWARMING AND FORMULATION OF STABLE MAGNETIC VITRIFICATION
SOLUTIONS

By

Eman Shreteh

August 2018

Chair: Carlos Rinaldi
Major: Biomedical Engineering

Cryopreservation by vitrification offers great promise in biobanking of tissues and organs, and it may be achieved using specialized solutions known as cryoprotective agents (CPAs). Nonetheless, current rewarming approaches present challenges in preserving structural integrity of bulk tissues due to thermal stress associated with devitrification and non-uniform heating. One novel approach to overcome these challenges is nanowarming, where superparamagnetic nanoparticles are added to CPA solutions, (magnetic CPA- mCPAs). Upon exposure to an alternating magnetic field (AMF), thermal energy dissipates from the particles. Consequently, rapid and uniform heating could be achieved in a volumetric fashion. Three CPA solutions: VS55, VS83 and DGPE were prepared and magnetic nanoparticles were dispersed at 1 mg Fe per ml of CPA. Particle stability in each formulation was assessed at room temperature over a two-week period. Then, samples were vitrified to LN$_2$ temperature followed by nanowarming in AMF of 20 kA/m and 360 kHz. Monitoring stability took place by obtaining hydrodynamic diameter ($D_h$) via Dynamic Light Scattering (DLS). Results showed excellent stability against aggregation in two CPAs. Next, comparison between two types of temperature sensors, fiber optic and thermocouple, was performed to evaluate their reliability and sensitivity in an electromagnetic environment. Results showed that cryogenic fiber optic probes were
reliable in measuring heating rates. Next, mCPA was prepared based on VS55 with nanoparticles at a concentration of 6 mg Fe per ml and nanowarming heating rate was evaluated. These studies indicated an initial heating rate of ~200 °C/ min upon application of AMF and that heating rates can be tuned through varying AMF amplitudes.
CHAPTER 1
INTRODUCTION

1.1 Current Challenges and Motivation

Despite advances in tissue and organ preservation, there is a severe tissue and organ shortage for transplantation. This is considerably affected by present tissue and organ preservation methods, which limit the time window between donation and transplantation. Cryopreservation by vitrification has great potential in revolutionizing tissues and organ biobanking, and extending shelf-life of biologics. Cryopreservation by vitrification is a non-freezing method that allows storage for a long time by either using ultra-fast cooling rates or highly concentrated solutes [10].

Preserving the structural integrity of vitrified bulk multicellular tissues is essential to maintaining their function. Devitrification and thermomechanical stress are the two main challenges of the conventional rewarming method, immersion in warm liquid baths. To overcome these challenges, rapid and uniform heating are critical. Although previous studies have investigated microwaves to achieve higher rewarming rates, their limited penetration depth resulted in non-uniform heating and thermal stress damage [31, 32].

Nanowarming is a novel volumetric heating approach where CPA solutions containing iron oxide magnetic nanoparticles (mCPA) are used to vitrify a perfused organ or immersed tissue specimen [4]. During rewarming, an alternating magnetic field (AMF) is applied to vitrified samples resulting in heat release by the nanoparticles. The large penetration depth of AMFs and the distribution of particles within the vasculature of the organ enable uniform heating. The rate of heating may be controlled through the magnetic properties of the particles, concentration of the nanoparticles in the mCPA, and the amplitude and frequency of the field applied [87].
Development of Nanowarming in the field of tissue and organ transplant is in its early stages. In fact, at the time of this writing, there are currently two published studies associated with translational aspects of nanowarming [20, 35]. Nonetheless, both studies present limitations as neither demonstrates preliminary aspects of prepared mCPAs such as stability of particles or thermal characterization. Furthermore, although one study illustrates heating rates and quantifies thermal energy dissipation of commercially-obtained particles, measured heating rates were not high enough due to aggregation of particles [35, 78]. Similarly, the other study did not demonstrate stability, magnetic characterization, nor quantified thermal energy dissipation [20]. This work aims to fill these gaps by formulating stable mCPAs, and investigating reliable temperature sensors in electromagnetic environment as well as measuring initial heating rates at LN2 temperature and quantifying energy dissipated upon exposure to AMF.

1.2 Objectives

The main objective of this work was to formulate colloidally stable vitrification solutions that achieve rapid rewarming rate. The following were the specific aims:

1. Develop novel mCPAs consisting of PEG-coated magnetic nanoparticles and assess colloidal stability of particles dispersed in CPAs solutions.

2. Compare sensitivity and reliability of electric and optical-based sensors in an electromagnetic environment by investigating offset interference.

3. Achieve rapid rewarming rate to prevent devitrification and quantify thermal dissipation of energy.

4. Demonstrate tuning of heating rate at various amplitudes of applied AMF to vitrified mCPA.
CHAPTER 2
BACKGROUND

2.1 Significance

According to data from the United Network for Organ Sharing (UNOS), approximately 20 people die daily while waiting on a transplant that they never receive and every 10 minutes, someone is added to the transplant waiting list [5]. The number of patients wanting a transplant far exceeds the supply of available organs as shown in Fig. 2-1 [5]. Bridging the gap between organ demand and supply would have prevented or postponed over 30-35% of all deaths in the U.S. per year [56, 57, 58]. Yet, many organs that could be used for donation are discarded [44]. For instance, more than 60% of donated hearts and lungs [66] and approximately 20% of donated kidneys [59] are discarded. Also, in the U.S., there are 185000 amputations performed yearly [4]. Thus, if a fraction of the healthy amputations is transplanted, it will improve upon quality of life of many individuals with limbs loss. The gap is attributed, in part, to the current organ preservation methods that preserve organs between donation and transplantation. For example, preservation of amputated limbs is 6 hours [76], and of hearts is 3-6 hours [4].

Biobanking of biologics extends their shelf-life by storing for long period of times, and progress in this field could revolutionize various aspect of medicine, scientific research, and public health. For instance, by expanding the lifespan of multicellular tissues, it allows for better pre-screening and matching procedures for immunotype and organ size. Further, it would also alleviate the economic burden associated with organ impairment, costs of hospital stay and transporting of organs from donor to recipient site. Last, advancements in biobanking would also impact fields of onco-fertility [4], tissue engineering, and regenerative medicine.
2.2 Methods of Tissue and Organ Preservation

**Hypothermic Preservation**

Static preservation is the most prevalent form of organ preservation at low temperatures, and it refers to perfusing the organ with preservation solutions followed by placing it in a sterile bag while being immersed in the solution, which is then placed in a container on ice [41, 45]. The purpose of these solutions is to maintain an appropriate biochemical environment while slowing down biological processes. Examples of these solutions include University of Wisconsin solution, Euro Collins solutions, and the Bretschneider solution [41]. While this method is advantageous due to ease of transport, inability to access organ during storage for further organ examination present a main limitation. Another form is hypothermic continuous perfusion. It refers to continuous pumping of fluids with nutrients and substrates at above 0°C storage [46].
While this method mimics physiology of the blood, it is limited by the impairment it causes the epithelial as well as metabolic changes related to effects of cooling [46].

**Normothermic blood perfusion**

The organ care system (OCS) is a new portable technology that is designed specifically for donor hearts and lungs [41]. It addresses some of the shortcomings associated with hypothermic storage, such as organ access during storage. It also extends duration of storage by maintaining hearts in a warm beating ex-vivo state [41]. Thus, OCS has potential in both storage and treatment. While this method extends perseveration time three additional hours, it requires relatively more resources than other methods. For instance, OSC requires 1.5 liters of donor blood in order to maintain hematocrit of 30% [41].

**Cryopreservation: Freezing and Vitrification**

Cryopreservation refers to storage at subzero temperatures, and it may be approached by either freezing or vitrification [46]. Freezing is regarded as reorganization of water molecules into ice crystals [10]. To reduce detrimental effects of freezing associated to vascular damage, cryoprotective agents (CPAs) are added. For instance, conventional cryopreservation of many cell types requires substituting 30% of cell water by CPAs followed by storage at a temperature that is lower than -100 °C [12]. The interrelationship between cooling rates and survival has been explained by the two-factor hypothesis by Mazur [13]. At low cooling rates, toxic solution effects such as toxicity take place since ice forms extracellularly raising osmolality. On the other hand, at high cooling rates, intracellular ice formation leads to cell death [16].

Applying conventional cryopreservation to multicellular tissues and organs jeopardizes structural integrity of tissues as ice formation damages vascularization as a result of extracellular ice formation that ruptures capillaries. In contrast, cryopreservation by vitrification is a non-
freezing solidification of a liquid in an amorphous state [7]. By suppressing crystallization, it allows bulk tissues to be vitrified without harmful effects of ice formation.

2.3 Cryopreservation by Vitrification

2.3.1 Background

The concept of cryopreservation by vitrification was originally thought of in terms of outrunning the kinetics of crystallization in living systems [10]. As water is cooled very quickly, there will be insufficient time for crystals to grow, so the system reaches a vitreous state [69]. This concept was pursued actively by Luyet in 1937, as he focused on samples that could be vitrified and rewarmed very rapidly, such as thin films [10]. Vitrification of living cells was first reported in 1968 by Luyet and Rapatz, showing cooling of erythrocytes in the presence of 8.6 M glycerol, which preserved cells from hemolyzing [70]. Then, many attempts followed focusing on using lower concentrations of CPAs to minimize toxicity while managing cooling and rewarining rates. Unfortunately, this was proven to be impractical for multiple reasons; one of which is lack of technology. This direction changed in 1981 as Fahy proposed a different approach that focused on utilizing high concentrations of solutes which lowers the critical cooling and rewarining rates needed [10]. He also focused on introducing these solutions at relatively higher temperatures as opposed to previous attempts, where CPAs were introduced at very low temperatures such as -55 °C [50, 42]. The objective of doing so was to allow usage of more compatible systems of organ perfusion [10]. Fahy’s work has been credited with changing the field of cryopreservation by following a more practical approach while focusing on prevention of ice formation and the mechanical injury associated with it. His efforts have also emphasized the ability to refine CPAs parameters, such a concentration, osmotic effects, temperature and addition and removal protocols of CPAs [10].
As cooling progresses and temperature is lowered, translational and rotational motion is halted, such that the system is trapped in a high energy state, resembling the vitreous state [12]. Hence, the fluid transitions to a molecularly arrested amorphous solid glassy state without any crystalline structures [45, 47, 85]. In general, vitreous cryopreservation may be achieved by either ultra-fast cooling or addition of highly concentrated solutes, or both [47]. During vitrification, viscosity changes by 12 orders of magnitude from a liquid-like material at room temperature to a solid-like material at storage temperature below the transition temperature [4].

### 2.3.2 Basis of vitrification

The glass transition temperature ($T_g$) is the temperature at which vitrification takes place [10]. Although $T_g$ is associated with a change in physical properties during vitrification, such as specific heat capacity, refractive index, and other mechanical properties, it is not a phase transition as liquid is cooled past its melting point [7, 16]. This contrasts with crystallization, which is a phase transition where liquids are cooled below the melting point resulting in latent heat release as water is transformed to a solid phase [7, 51]. In vitrification, as cooling proceeds, the energy of the molecules gradually decreases despite the disorderly state at which molecules remain at since liquid is too viscous to flow. Viscosity is inversely proportional to cooling rates and increases exponentially with decreasing temperature [11-12], and so viscosity outruns the kinetics of ice formation [10]. Viscosity profile of one vitrification solution, VS55 solution, is illustrated in Eq. (2-1) [11].

$$\mu = \begin{cases} 
1.21 \times 10^4 & - 100^\circ C < T \\
4.2710^{-23} e^{-0.6097T} & - 140^\circ C < T < -100^\circ C \\
4.63 \times 10^{14} & T < -140^\circ C
\end{cases}$$

(2-1)

Thermodynamically, vitrification occurs during cooling as homogenous nucleation temperature ($T_h$) is depressed to $T_g$ while CPAs interact with water molecules. This prevents
water molecules from interacting with each other to form ice crystals. Consequently, water remains in an arrested liquid state. This is illustrated in the phase diagram of a binary system during cooling a propane-1,2- diol/ water as shown in Fig. 2-2 [12]. For instance, in the concentration region of 35%- 40%, ice nuclei may appear in that region, and if warming rate is not rapid enough, then devitrification will occur as indicated by $T_d$. Above 50% concentration, the system will vitrify and devitrification will not be detected, hence the system may be regarded as stable. In fact, as the $T_h$ curve meets the $T_g$ curve, the system may be cooled slowly to reach $T_g$ without ice formation. Also, the intersection between $T_g$ and $T_m$ provides the minimum concentration needed to achieve vitrification, also known as concentration needed to vitrify (Cv) [12].

![Phase Diagram](image)

Figure 2-2. Path followed temperature vs concentration plot of propane-1,2- diol/ water phase diagram. $T_h$ is the homogenous nucleation temperature, $T_g$ is the transition glass curve, $T_m$ is the melting point curve, and $T_d$ is the devitrification [12].

2.3.3 Cryoprotectant Solutions (CPAs)

Addition of CPAs have both kinetic and thermodynamics effects. Kinetically, addition of CPAs increases viscosity of the sample to achieve vitrification while thermodynamically, it
lowers melting point and homogenous nucleation temperatures of the sample. Without CPAs, cooling rates for small tissues may be around $10^7 \, ^\circ C/\, \text{min}$ [7]. Mechanisms in which CPAs preserve biological integrity may be by interacting with water molecules through hydrogen bonding [10, 51, 76] as well as interacting with the cell membranes to protect against denaturation. CPAs may be single or multiple [47]. They may also be classified into permeating and non-permeating components [64]. Permeating components penetrate cells intracellularly to prevent ice crystals and membrane rupture. Examples of penetrating components include DMSO, PG, EG and Glycerol. On the other hand, extracellular components are non-permeating that reduce osmotic stress across cell membranes. Examples of non-permeating include sucrose, trehalose, PVP and dextrose.

CPAs that enable vitrification may be called vitrification solutions (VS)/vitrificants [10], which may be defined as cryoprotective solutions that promote amorphous solid state rather than crystallization [79, 85]. One type of CPAs are ice blockers which are used in vitrification and not freezing [10]. They are molecules that prevent or reduce ice nucleation or growth through specific interactions with ice or ice nucleating agents [10]. Another type is antifreeze proteins (AFP), also known as thermal hysteresis proteins (THP), which suppress formation and growth of ice crystals by lowering the freezing point of aqueous solution a non-colligative fashion [4, 46]. AFP are a form of ice blockers such that they function in a similar fashion to prevent ice from growing by adsorbing to the surface of ice crystals [10].

Carrier solution is the medium in which CPAs are dissolved in, and may contain pH buffers, osmotic agents and salts. Therefore, it is considered a physiological medium to maintain the tonicity of cells once added to cells. Example of carrier solutions include LM5 and PBS buffer. Parameters considered when selecting CPAs include concentration, temperature, timing,
and osmotic effects of each step of CPA introduction and removal. On a cellular level, controlled addition and removal protocols are necessary to prevent toxicity, cell lysis, and differentiation. Delivery of CPAs to bulk specimens may be influenced by viscosity, concentration, delivery protocol, perfusate selection, and permeability [85]. The effectiveness of CPAs may partially depend on their solubility of water [15].

2.3.4 Applications: Cells, Tissues and Organs

Isolated cells, homogenous cell populations, are relatively easy to cryopreserve, and there are many vitrification protocols that have been developed; some of which utilize minimum vitrification volume methods [29]. Examples of cells for which vitreous cryopreservation protocols are used include Hematopoietic stem cells [10], endothelial cells [64], chondrocytes [52, 54] and mesenchymal stem cells [20]. Furthermore, it has proven successful to vitrify small macroscopic tissues. For instance, currently, small ovaries, blood vessels, heart valves, cornea, liver slices [47, 84], skin, cartilage and tendon [8] are the only macroscopic structures that have the capacity to recover after vitrification.

Articular cartilage: Vitreous cryopreservation of articular cartilage has been extensively studied [47, 52, 54-55] since current preservation methods include hypothermia storage at 4°C for 28-84 days; nonetheless, tissue deterioration commences after 7-14 days [55]. Therefore, progress in biobanking of articular cartilage may allow for better surgical planning as well better size matching since osteochondral allograft transplantation is the recommended intervention to treating larger lesions of arthritis [55]. Examples of studies that have been performed include examining of osteochondral plugs of porcine [54] and sheep [47] origin. In such studies, gradual infiltration of pre-cooled CPA was performed till final concentration was reached. This was followed by pre-cooling samples then storing them in a bath containing isopentane in a mechanical freezer, then samples were removed from the bath and stored at -135°C [54].
Rewarming was achieved by slow warming followed by rapid rewarming rate where samples were placed to rewarm by immersion in water bath containing 30% DMSO at RT. Removal of CPA solution was in a reverse pathway to addition protocol by washing out solution gradually [54]. Examples of CPA solutions used include VS55, VS83 and DGPE. Furthermore, permeation to thick intact articular cartilage in relation to increased concentration and toxicity of CPAs has been studied [55].

**Heart valves and vessel segments:** Many studies have shown success in vitrification of heart valves of different animal models, such as pig heart valves [65], as well as in vivo preclinical studies of heart valve allografts [60]. Furthermore, studies of thermal expansion of vessel segments have also been carried out, investigating permeation with various CPA solutions such as DMSO, DP6 and VS55 [62, 63] Additionally, other macroscopic tissues have been studied such as nerve graft [8, 73], myocardium, skin and cornea [65]. Vitrification of the latter, myocardium, skin and cornea, have been examined by fully immersing tissue in VS55 with stepwise increase in concentration at 4°C.

**Vascularized composite allografts:** Vitreous cryopreservation of composite tissue is least investigated due to technical challenges of re-implantation and attachment. In one study [76], feasibility to re-implant cryopreserved limbs was examined in a rat model by comparing two groups, each of six: above- knee amputation and Syme’s amputation. This was done by using 10% DMSO that was perfused at 4°C followed by storage in LN2 for 15 days [76]. Rewarming was achieved using a 40°C water bath for eight minutes followed by perfusion buffer to drain out CPA. [76]. Results showed successful re-implantation in the Syme’s group whereas all re-implantation in the above knee group had failed. The latter was indicated by swelling that led to embolism ceasing blood flow [76]. This study did not provide any structural nor functional
evaluation of re-implanted limbs as it was based on observation. Furthermore, this study did not examine potential devitrification in the selected concentration of CPA. This could have potentially provided better insight to the cause of failed re-implantation. Another study in composite tissue was cryopreservation of epigastric flaps in rats [39]. Last, a successful re-implantation of an amputated finger post storage in LN2 for 81 days has been also demonstrated [76].

**Kidney:** Vitreous cryopreservation of kidneys has been one of the most studied settings. One study of vitrified rabbit kidneys at -45°C that have survived post vitrification and were transplanted to original donors [16, 85]. This was using M22 solution, which is a propriety solution of Alcor Life Extension Foundation with concentration of 9.345 M [93- 94] that was administered to the kidney at -22 °C at 80 mmHg for 25 minutes [16, 85]. This study marked a historical case as it was the first successful vitrification of a large solid organ. Another study was using 7.5 M concentration of the cryoprotectants of VS4 was perfused kidney at -3°C with high pressure [19]. Results showed immediate functional recovery. Nonetheless, vitrification of VS4 was only attainable under higher hydrostatic pressure, which promoted using an 8.4M concentration of the cryoprotectants of VS41A (also known as VS55) solution to vitrify at 1 atm [86]. Results showed failure in survival post transplantation and delayed graft function [86]. Also, while using VS41A, devitrification posed a challenge as critical rewarming rate to prevent ice formation was estimated 40- 270 ºC/ min [25]. Last, other attempts have aimed to vitrify kidney precursors [84] and kidney slices [88].

**Liver:** Liver is believed to be one of the most complicated organs to vitrify [46]. In addition to preserving livers for transplants, there is a need to preserving its integrity for drug metabolism and toxicity studies [88]. A recent study happened to be a comparison between rapid
freezing and vitrification on preservation of liver and kidney slices of rats and [88]. In this study [88], two solutions were examined including 7.5M and 8.4M concentrations of cryoprotectants of VS4 and VM3, respectively which were introduced and removed in a stepwise manner. Rewarming modality included immersion in a warm water bath. One of the most studied methods attempted to preserve and store livers at low temperature is directional solidification in animal models such as porcine, murine and rats [10, 77, 83].

**Heart:** Hearts present one of the first attempted organs to be vitrified in 1970 [10]. This was done by using frog hearts that were cooled to -79°C using 11M of ethylene glycol showing good recovery [10]. Nonetheless, it was reported that hearts could not withstand chemical toxicity as they could not tolerate more than 5 M EG [49]. Another attempt was using 10% Glycerol to freeze two rabbits’ hearts at -21°C [46]. Another happened to be using 15- 20% DMSO to be perfused in murine hearts at -15 °C and stored for 70 minutes [46]. One of the recent attempts was a re-implantation of preserved rodent hearts using Antifreeze proteins and University of Wisconsin solution at sub-zero temperatures at -1.3°C [89]. Although many studies have been performed in vitrifying hearts, several challenges are hindering the success of vitrification and the recovery of the structural integrity of vitrified heart.

### 2.3.5 Challenges

Structural and functional recovery of vitrified complex tissues and organs has been challenging despite improvements in CPA solutions and administration protocols. This is related to tissue-dependent properties of low thermal conductivity, diversity, and heterogeneity of cell types and densities within the organ [4]. According to the Organ Banking Summit [4], there are six challenges associated with complex tissue and organ preservation that may be summarized as follows:

1. Revival and repair protocols
2. Ischemia and reperfusion injury
3. Chilling injury
4. Devitrification
5. Thermomechanical stress
6. Toxicity

To a large extent, three of these challenges are related to current rewarming methods including devitrification, thermomechanical stress, and chemical stress, which will be discussed next.

**Devitrification:** Devitrification refers to ice formation during rewarming post vitrification [10]. Ice growth follows formation of ice nuclei that had initially formed at temperatures that are too low for ice growth [8]. While growth of nascent ice crystals relies on diffusion of water, ice nucleation depends on local molecular motion [10]. This elucidates the occurrence of nucleation below passing T_g, which happens at lower rate [7, 10]. Furthermore, the thermal conductivity of the glassy state may be an order of magnitude lower than of the crystalline state [9].

During rewarming, as the system’s temperature range is near T_g, crystallization may be resumed which occurs quickly unless rewarming rate is very rapid to prevent this process. Hence, the critical rewarming rate is found to be higher than the critical cooling rate. For instance, for a binary system of ethylene glycol/ water, there is approximately 5- log increase in critical rewarming rate as critical cooling rate is increased from 10 °C/ min to 100 °C/ min [10] illustrated in Fig. 2-3 [48, 50]. Critical rewarming rate is the rate at which ice formation is not observed while critical cooling rate is the rate at ice crystals are suppressed [6]. Formation of ice during rewarming contributes to not only damaging effects associated with ice but it also contributes to non-uniform heating causing thermal stress that leads to fracture since the vitrified
organ is brittle [12]. Thus, in general, critical rewarming rates are typically two or more orders of magnitudes greater than critical cooling rates [7, 24].

Figure 2-3. Critical cooling and rewarming rates of various concentration of ethylene glycol in water [48, 50].

Chemical stress: Using high concentrations of CPAs is necessary to suppress ice formation during cooling and to reduce the critical rewarming rate. Yet, using high concentration leads to chemical stress on the vitrified specimen that is associate with both toxicity and osmotic stress. Chemical toxicity of CPA is temperature dependent, and it is also related to water/ CPA interaction. The higher the cooling rate, the shorter the exposure time and so toxicity is reduced [27]. Furthermore, there is a correlation between hydrogen bonding and toxicity, such that CPAs with polar groups that interact weakly with water at higher concentration are less toxic [7]. Although effects of toxicity in damaging the cell membranes have been attributed to osmotic stress, oxidative damage and metabolic disruption dehydration, destabilizing and denaturing
proteins [27], their mechanisms are yet to be known [7, 17]. Osmotic stress has been limited in current vitrification protocols of small macroscopic tissues by allowing 10-15 minutes during stepwise loading and removal of CPAs to achieve osmotic equilibrium [65].

The objective of using high concentrations in CPAs is to suppress ice crystallization while using attainable cooling and rewarming rates that are dependent on available technologies. Nonetheless, as a result of using high concentrations, toxicity presents a major obstacle in achieving successful vitrification as more than half of water within the specimen is replaced with solutes. As mentioned above, rewarming rates are at an order of magnitude higher than cooling rates. Since there are no current rewarming technologies that are able to achieve the critical rewarming rate, higher solute concentrations are used to decrease critical cooling rate necessary to vitrify tissue which in turn reduce rewarming rate. By increasing concentration, toxicity becomes an impediment that causes tissue damage. Current vitrification protocols attempt to mitigate effects of toxicity by administration of cooled CPAs at low temperature while increasing concentration either via gradual perfusion or stepwise immersion. Other protocols may continue to cool down the system during CPAs administration taking advantage of temperature-toxicity dependence [12]. To conclude, toxicity is a multidimensional facet that incorporate carrier solution, temperature at which CPAs are administered and removed, and exposure time [4]. Thus, ability to achieve higher rewarming rates may combat the need to use high concentrations, potentially addressing toxicity concerns.

**Thermo-mechanical stress:** Volume change is proportional to temperature change. As temperature decreases, CPAs contract occupying a smaller volume [10]. This induces mechanical stress during vitrification that may be referred to as thermomechanical stress (TMS), which is described by thermal expansion coefficient as shown in Eq. (2-2).
\[ \alpha = \frac{1}{V} \left( \frac{\partial V}{\partial T} \right)_P \quad (2-2) \]

Where \((T)\) is temperature, \((V)\) is the volume measured at a constant pressure \((P)\), and \((\alpha)\) is thermal expansion coefficient, which is characteristic of a particular material.

TMS plays a key role in preserving structural integrity of the vitrified organ and intensifies as the sample size increases, making it critical in multicellular bulk tissues [4]. TMS is driven by differential thermal expansion, and it is path – dependent in the sense that the events samples experience up to the point of interest dictate the level of TMS [4]. To illustrate, during cooling stages, temperature distribution varies within the specimen encompassing a non-uniform profile. In other words, the glass transition temperature is passed at different times within the specimen during vitrification [7, 10]. The organ also experiences additional events during vitrification protocol including concentration of administered CPAs, and so the system is more susceptible to TMS by the time rewarming takes place as it had already accumulated and perhaps caused irreversible cellular changes at prior stages of the process. Consequently, to prevent further damage of fracture, a uniform temperature distribution becomes imperative. Another, adherence to container’s walls may also impose stress on the samples causing TMS within samples [24]. Last, as mentioned above devitrification also induces TMS.

In summary, there are tissue- specific factors that induce TMS during rewarming and contribute to both mechanical stress and fracture formation, which ultimately jeopardize the structural integrity of the vitrified organ. These factors are [4, 12, 24]:

- Heterogenous material properties
- Non-uniform temperature distribution
- Devitrification
- Adherence to container’s wall
2.3 Nanowarming Phenomena

2.4.1 Current Rewarming Modalities

Conventional rewarming is achieved by surface conduction where vitrified samples are immersed in a warm water bath. However, as discussed in Section 2.3.5, rapid and uniform heating are not achieved using such methods. This contributes to poor structural and functional recovery due to formation of ice and TMS. Accordingly, an alternative approach is rewarming tissues from within in order to achieve more uniform heating. Methods of microwave warming have been studied in warming of canine kidneys, but limitation in penetration depth lead to uneven heating profile since this method relies on absorption properties of CPAs and tissues [24, 32, 34]. Furthermore, other approaches of electromagnetic radiofrequency using an applied frequency have been sought [4, 31, 36]. Nonetheless, effects of hotspots led to unfavorable outcomes of non-uniformity [24].

One novel approach is using magnetic nanoparticles, nanowarming, which was first suggested in 2014 [71]. Nanowarming is essentially adding biocompatible superparamagnetic iron oxide nanoparticles to CPAs, forming magnetic-CPAs (mCPAs), that could be either perfused into organ or added into solutions that surround a specimen [4] as illustrated in Fig. 2-4 [71]. Biocompatibility of IO MNP ensures their safety [43]. Nanowarming is advantageous relative to other approaches because heating is dependent on particle properties and distribution within the sample, since frequencies of magnetic field interact negligibly with tissue as opposed to depending on tissue properties. For instance, perfusion of mCPAs through an organ, energy release may be initiated upon exposure to an AC magnetic field at a solid state of low storage temperature (i.e. -196°C) as opposed to other methods of electromagnetic warming that are ineffective since heat is delivered most effectively to tissues that are rich in polar liquids [4]. In
addition, nanowarming offers the ability to regulate heating rate by tuning properties of the nanoparticles, their concentration, and the amplitude and frequency of the applied AMF. The distribution of the nanoparticles within organs and vascularized complex allografts might be assessed via using magnetic resonance imaging (MRI) or through magnetic particle imaging (MPI) [80-81]. Furthermore, distribution of heating profiles may be controlled using magnetic selection magnetic field gradients, which have been demonstrated theoretically and experimentally [90-91]. Last, non-invasive imaging modalities may be used to monitor local temperature and heating rate of the nanoparticles [74, 82].

Figure 2-4. Rewarming of vitrified tissues using nanowarming for bulk multicellular tissues provides rapid and uniform heating, so it ameliorates devitrification and fracture [71].

2.4.2 IO MNP

Superparamagnetic nanoparticles are single-domain nanoparticles that consist of magnetite (Fe₃O₄). Using IO MNP, heating is dependent upon the distribution of the particles. Heating proceeds by using a helical coil to apply radiofrequency magnetic field, and magnetic energy is absorbed and converted into thermal energy [1, 30]. Two dissipation mechanisms determine total absorption of AC magnetic field energy [14] illustrated in Fig. 2-5 [68]. One is
Neel relaxation, which is the internal rotation of a magnetic moment, and so thermal energy is dissipated through rearrangement of atomic dipole crystals [30, 68] Eq. (2-3). The second is Brownian relaxation, which is the external physical rotation of the particles, and so through shear stress, thermal energy is delivered to the surrounding media [30, 68] Eq. (2-4).

\[
\tau_N = \tau_o \exp \left( \frac{K_A V}{k_B T} \right) \tag{2-3}
\]

\[
\tau_B = \frac{3\eta V_H}{k_B T} \tag{2-4}
\]

\[
\tau = \frac{\tau_N \tau_B}{\tau_N + \tau_B} \tag{2-5}
\]

Where \((\tau_o)\) is pre-exponential decay factor, \((K_A)\) is the anisotropy energy, \((V)\) is a particle volume, \((k_B)\) is Boltzmann’s constant, \((T)\) is temperature, \((\eta)\) is the viscosity of the carrier medium, \((V_H)\) is the hydrodynamic diameter, and \((\tau)\) is the effective relaxation time combining the contribution of both mechanisms.

![Diagram of Neel and Brownian rotation](image)

Figure 2-5. Mechanisms of magnetic nanoparticles relaxations upon exposure to AMF [68].

### 2.4.3 Relevant Nanowarming Studies

At the time of writing this thesis, there are only two translational applications of nanowarming [20, 35]. Yet, there have been several publications on nanowarming phenomena, including a feasibility study [23], and numerical simulation of heating profiles [21]. In addition,
optimized heating efficiency studies investigating concentrations of two types of magnetic nanoparticles have also been published [26]. Last, analysis of thermomechanical stress in volumetric rewarming using IO MNP has been compared to surface-based heating by providing average warming rates and temperature differences at different points at the center and the surface of the vitrified specimen [24].

One translational study showed vitrification of Mesenchymal stems cells using CPAs composed of PG, EG and trehalose [20]. Thus, it contained a non-permeating solute, trehalose. Particles were prepared via co-precipitation chemical synthesis. Concentration of particles in the prepared CPAs ranged between 0.01-0.1% in CPAs, and basic characterization of hydrodynamic and magnetic diameter were presented in the study. However, there was no assessment of particles’ stability in the prepared suspension. Furthermore, there was no characterization of thermal energy dissipation nor rewarming rate. Another translational study used commercially-obtained IO MNP, which were coated with a mesoporous silica shell followed by combined PEG and trimethoxysilane [35]. Particles were suspended in VS55 and used to vitrify fibroblasts, vessel segments, and heart valve leaflets followed by nanowarming at 20 kA/m and 360 kHz AMF [35].

VS55 solution has been investigated greatly through the last two decades. This includes both its physical properties as wells as vitrification of macroscopic tissues such as articular cartilage and vessel segments. Last, one study of heating rate assessment of prepared magnetic VS55 (mVS55) has been performed. This was using 1 ml of VS55 loaded with 10 mg Fe/ml in a 20 kA/m magnetic field at 360 kHz, which led to a heating rate of 200 °C/min below T_g of VS55 (-123°C) and 100 °C/min above T_g [4].
CHAPTER 3
MATERIALS AND EXPERIMENTAL METHODS

3.1 Materials

Three mixtures of CPA solutions were prepared: VS83 [10], VS55 [10] and DGPE [52]. A summary of their concentration and component compositions are provided in Table (3-1). Reagents of these mixtures were obtained from Sigma- Aldrich. The mixing of VS55 and VS83 solutions was prepared in accordance with the heart valve cryopreservation media protocol [10], which includes the sequence in which individual reagents were added as well as the filter type used.

Table 3-1. Concentration and composition of CPA solutions.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>55% w/v (8.4M)</td>
<td>83% w/v (12.6M)</td>
<td>47.7% w/w (6.5M)</td>
</tr>
<tr>
<td>Components</td>
<td>3.10 M DMSO</td>
<td>4.65 M DMSO</td>
<td>18.0% w/w DMSO</td>
</tr>
<tr>
<td></td>
<td>2.21 M PG</td>
<td>3.30 M PG</td>
<td>14.1% w/w Glycerol</td>
</tr>
<tr>
<td></td>
<td>3.10 M Formamide</td>
<td>4.65 M Formamide</td>
<td>5.8% w/w PG</td>
</tr>
<tr>
<td></td>
<td>2.4 g/L HEPES</td>
<td>2.4 g/L HEPES</td>
<td>9.5% w/w EG</td>
</tr>
<tr>
<td></td>
<td>20% v/v PBS 1X</td>
<td>20% v/v PBS 1X</td>
<td></td>
</tr>
</tbody>
</table>

Superparamagnetic iron oxide nanoparticles were synthesized via chemical synthesis of co-precipitation [33, 92], then coated with a layer of Oleic acid. This followed by ligand exchange between oleic acid coating and PEG- silane using silane coupling agents resulting in covalently- grafted PEG- coated iron oxide particles [37- 38]. Particles were prepared in the Rinaldi Lab (Courtesy of Andreina Chiu Lam).
3.2 Methods

3.2.1 Characterization

3.2.1.1 Dynamic Light Scattering

With the objective to assess stability of particles in CPA solutions at different conditions and time points, hydrodynamic size of particles was determined via Dynamic Light Scattering (DLS) measurements using a Brookhaven Instrument BI-90 Plus Dynamic Light Scattering analyzer. This measurement is based on fluctuation of scattered light of particles in suspension, which are detected and analyzed using correlation function. Scattering of light within the sample is caused by the Brownian motion of particles dispersed in a medium. This is a result of the thermal energy of solvent molecules have as it is induced by their bombardment. Thus, when the light from the 15mW laser source illuminates the sample, fluctuations of the intensity of the scattered light depend on the size of the particles [60]. Analysis of velocity fluctuations of the scattered light is analyzed, and a detector is used to collect the scattered light. The detected intensity is then used to measure the autocorrelation function such that digital autocorrelator processes the signal in relation of the exponential decay, where the decay constant is proportional to diffusion coefficient. Once diffusion coefficient ($D$) is extracted, it is then used in Stokes Einstein Eq. 3-1 to calculate the hydrodynamic diameter ($D_H$),

$$D_H = \frac{k_BT}{3\pi\mu D}$$  

Where ($k_B$) is Boltzmann’s constant, ($T$) is the temperature, ($\mu$) is viscosity of media, ($D_H$) is the hydrodynamic diameter of the particles and ($D$) is the translational diffusion coefficient, which is obtained from the decay correlation function.

Hydrodynamic diameter refers to the way a particle diffuses within a fluid. Thus, the obtained diameter of the particle is in reference to a sphere with a similar translational diffusion...
coefficient corresponding to the core and shell surface. Intensity weighted multimodal size
distribution function is generated, which is then used to calculate the volume weighted and
number weighted distributions via regression. Histogram of obtained diameters were then fitted
either to a lognormal distribution using Eq. (3-2) or binomial lognormal distribution using Eq.
(3-3).

\[ f(D_p) = \frac{A}{\ln \sigma_g D_p \sqrt{2\pi}} \exp \left(-\frac{\ln^2 \left( \frac{D_p}{D_{pgA}} \right)}{2\ln^2 \sigma_{gA}} \right) \]  (3-2)

\[ f(D_p) = \frac{N \cdot \varphi_A}{\ln \sigma_g D_p \sqrt{2\pi}} \exp \left(-\frac{\ln^2 \left( \frac{D_p}{D_{pgA}} \right)}{2\ln^2 \sigma_{gA}} \right) + \frac{N \cdot (1 - \varphi_A)}{\ln \sigma_g D_{pgB} \sqrt{2\pi}} \exp \left(-\frac{\ln^2 \left( \frac{D_p}{D_{pgB}} \right)}{2\ln^2 \sigma_{gB}} \right) \]  (3-3)

Where \( D_{pg} \) is the median diameter, \( \ln \sigma_g \) is a geometric deviation and A is a normalization
constant [61]. In both Eq. (3-2) and Eq. (3-3), A, B subscripts present the two populations in the
distribution, and \( \varphi_A \) is fraction of particles in population A with certain size. Last, \( f(D_p) \) is the
probability density of the particles. Once fitted, median diameter and geometric standard
deviation of volume-weighted distributions may be converted to mean hydrodynamic diameter
and arithmetic standard deviation using Eq. (3-4) and Eq. (3-5), respectively.

\[ D_p = \exp(\ln D_{pg} + \frac{\ln^2 \sigma_g}{2}) \]  (3-4)

\[ \sigma = D_p \sqrt{\exp(\ln^2 \sigma_g - 1)} \]  (3-5)

Where \( D_p \) is the mean hydrodynamic diameter and \( \sigma \) is the arithmetic standard deviation. In
this thesis, DLS measurements are presented in terms of volume-weighted distributions such
that mean hydrodynamic diameter and arithmetic standard deviation are provided. Last, light is
incident at a fixed angle of 90˚, temperature used was 25°C and selected parameters of fluid’s viscosity, and refractive index were media-specific.

3.2.1.2 Rheology

Viscosity measurements of each CPA mixture were obtained to be used in DLS size approximations. The instrument used was the Rotational Anton Paar MCR301 Rheometer. This technique is based on loading samples into a certain geometry of two-part components sandwiching the fluid such that upon its rotation, a shear flow is imposed on the fluid. In here, a Cup and Bob geometry was used as shown in Fig. 3-1. The temperature of both system and sample was controlled and maintained at 25°C. Torque that results from the viscous drag is measured by a sensor in the bob’s drive, which correlates with shear stress, whereas shear rate correlates to the rotational speed (angular velocity) while factoring in the gap between the two parts. Two cycles of increasing and decreasing shear rates were performed followed by calculating viscosity using Eq. (3-6). As shear rate is increased, torque is also increased and vice versa.

$$\mu = \frac{\tau}{\dot{\gamma}}$$

(3-6)

Where (\(\mu\)) is viscosity, (\(\tau\)) is shear stress and (\(\dot{\gamma}\)) is the shear rate.

Figure 3-1. Schematic Cup and Bob geometry [40].
3.2.1.3 SQUID Magnetometer

A Superconducting Quantum Interference Device (SQUID) was used to characterize magnetic diameter of IO MNP. Magnetization was obtained as a function of applied field at 300K, and it was normalized based on estimated mass of iron oxide sample, which was suspended in water. Working principle is based on placing sample between superconducting coils while applying high magnetic field. This induces electric current in the pickup coil. This current is then detected and converted to voltage, which is proportional to the sample’s magnetization. Data was fitted using non-linear regression to the Langevin- Chantrel model to estimate mean magnetic diameter.

3.2.1.4 Thermal Energy Dissipation

Energy dissipation rate of magnetic nanoparticles due to magnetic moment relaxation was quantified using the Specific Absorption Rate (SAR) [72]. SAR may be defined as energy dissipated per unit mass of magnetic nanoparticles Eq. (3-7). An alternating magnetic field (AMF) was generated in the coil using the EASYHEAT 8130L 10 kW induction heater. Hence, current is generated causing heating of samples.

\[
SAR \left[ \frac{W}{g_{Fe}} \right] = m_{sample} \times \frac{C_p}{m_{Fe}} \times \frac{\Delta T}{\Delta t}
\]

(3-7)

Where \((C_p)\) is the heat capacity of the media and \(\frac{\Delta T}{\Delta t}\) is the temperature rate increase.
3.2.1.5 Temperature Sensors

Two types of temperature sensors: thermocouple and fiber optic, were examined at various conditions of electromagnetic environment. While TCs are more cost effective, allow usage of multiple channels, respond fast and may be fabricated easily, they may be more susceptible to noise and AMF interference. Thus, to ensure reliable heating rates quantification of nanowarming, temperature vs time profiles of TCs, an electronic-based temperature sensor, were obtained and compared to FO, an optical-based temperature sensor, temperature measurements. The objective was to assess whether TCs can be used to monitor sample temperatures during vitrification and nanowarming to measure heating rates and calculate SAR. Fig. 3-2 shows tips of various temperature probes that were assessed in this work.

![Temperature sensor probes](image)

Figure 3-2. Tips of temperature sensor probes. (A) Fiber optics. (B) cryogenic fiber optics. (C) thermocouple type T.

<table>
<thead>
<tr>
<th>Type</th>
<th>TC_T</th>
<th>TC_E</th>
<th>FO</th>
<th>Cryo FO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature range [°C]</td>
<td>-250 to 350</td>
<td>-200 to 900</td>
<td>-80 to 200</td>
<td>-270 to 80</td>
</tr>
</tbody>
</table>

**Thermocouple**

Thermocouples (TCs), also known as thermoelements, are composed of two dissimilar metals that are joined at one end, referred to as the measurement (hot) junction. Wires do not join at the other end, instead they form a reference (cold) junction as they are connected to a signal
conditioning copper circuitry. The working principle of TCs is based on generating voltage difference. The dissimilarity between the wires connected at the junction generates voltage following the Peltier effect. Once temperature changes, this leads to change in voltage utilizing Thompson effect. Together, both combined effects is referred to as Seebeck effect, which relates voltage generated to the temperature difference. Thus, TCs do not provide absolute temperature since measurement difference between both junctions is converted to an electrical signal.

Difference in thermocouples is due to different combinations of materials that ultimately dictate TCs performance. In presented work, two types of thermocouples were examined including Type T (TC_T) of Copper/ Constantan and type E (TC_E) of Nickel- Chromium/ Constantan, both of which are 40 Gauge from OMEGA Engineering. Also, National Instrument Data Acquisition (NI-DAQ) (cDAQ- 9178) was used to process and store signals. In this thesis, two versions of TC_T were tested such that one was purchased then made in the lab while the other one was ready- made kit.

Making a thermocouple in the lab was by first removing the insulation layer, Pfa Teflon Jacket, from the both wires at approximately 2 cm. Upon removal, exposed wires are twisted for 4-6 turns creating. Whereas using the ready- made TCs, there was no need to perform the preceding description. Comparison between both was performed to check the impact of bonding on TCs sensitivity and response to AMF.

**Fiber Optics**

In here, two types of non- metallic sensors from Qualitrol were used: fiber optics and cryogenic fiber optics such that they differ in the temperature range as summarized in Table (3-2). The probe’s working principle is based on utilizing properties of light absorption and transmission in a semiconducting crystal, Gallium Arsenide (GaAs) as shown in Fig. (3-3). A
white light source is coupled into the fiber, and travels down to the fiber where some of it is absorbed by the crystal. Remaining unabsorbed light is reflected by a dielectric mirror directed back to the spectrometer. Variation of temperature affects the semiconductor’s energy band gap. This leads to an absorption shift, which relates transmission (unabsorbed) light to a specific wavelength. Thus, transmission spectrum varies with respect to a certain wavelength of unabsorbed light in response to temperature change, and so absolute temperature is measured as opposed to measurements using TCs where temperature is measured to the reference junction.

![Schematic of FO sensor](image)

Figure 3-3. Schematic of FO sensor [67].

### 3.2.2 mCPA Formulation

Three magnetic vitrification solutions (mVS) were prepared: VS55, VS83 and DGPE. Preparation of magnetic VS55 and magnetic VS83 was via sequential addition of components while using stir bar in a beaker. Components were added in the following order starting with PBS 1X as the first component followed by HEPES, PG, Formamide, DMSO and DIW. This was based on the heart valve vitrification protocol [10]. Once water is added, stirring both mixtures for 10 minutes was done followed by using 200 nm filters to ensure removal of any particulates [10]. Euro Collin solution was used as the carrier solution in the referenced protocol such that 200 ml for each 1 ml of media prepared; however, when preparing both VS55 and VS83, Euro Collins was replaced with PBS 1X at 20% v/v for media prepared.

Adding particles to the prepared CPAs started by concentrating the particles in DIW then they were added following. First, starting with 4 samples of 1.05 mg Fe per ml of PEG 2000- coated
particles suspended in DIW that were then transferred into a 1.5 ml 100 kDa Amicon filter. Next, samples were centrifuged at the following conditions: 7500g, 10 minutes at 25°C. Once concentrated, 0.5 ml of the prepared CPA was added to the concentrate (particles) followed by multiple pipetting to ensure good mixing. This mixture was then aspirated to a 4ml vial and another 0.5 ml of the prepared CPA was added to the filter device to ensure collection of any remaining particles. After several resuspensions, remaining amount was transferred formulating 1 ml of each mCPAs. Last, samples were then filtered using 0.2 µm nylon filter to ensure removal of any aggregates illustrated in Fig. (3-4). Specific component densities are provided in Appendix A.1.

Figure 3-4. Formulated 1 ml of three mCPAs and PBS 1X of particles suspension.

3.4.2 Colloidal Stability

Once mCPAs are formulated, two preliminary studies were performed to assess stability of the particles and ensure no aggregation nor precipitation. First, colloidal stability was assessed over a two-week period at room temperature where DLS data was collected on day 1, day 2, day 6, and day 14. Next, stability of particles’ suspension was assessed pre-vitrification and post nanowarming. This was done by transferring 300 ml of each sample into a cryogenic vial on day 6 where samples were then immersed in LN$_2$ to be vitrified for 1 week. Then, on day 14, rewarming of mCPA media took place by subjecting samples to an AMF of 321.3A and 276 kHz using the six-turn Pontine coil. Fig. 3-5 shows mVS55 before vitrification and post nanowarming. DLS data was collected prior vitrification on day 6 and post nanowarming on day
14. For all DLS measurements in this study, Quartz cuvettes were used such that all samples were loaded and diluted to 1 ml in 1:10 dilution ratio in the same media. Since DMSO is a common component among all formulated CPAs, its refractive index used was 1.478.

Figure 3-5 An overview of pre-vitrification and post nanowarming stability study. (Left) mVS55 during IH. (Top) vitrified mVS55 as sample was taken from LN2. (Bottom) mVS55 post nanowarming.

3.4.3 Rheology measurements

The viscosity of prepared CPAs was measured in a Rheometer instrument. This was by preparing 7 ml of all four solutions without particles: VS55, VS83, DGPE and PBS 1X. Two cycles of increasing and decreasing shear rates were performed for each mixture at room temperature using Cup and Bob geometry. Values obtained were used to obtain hydrodynamic size throughout colloidal stability studies.

3.4.4 Applied Magnetic Field and Temperature Sensors

Reliable measurements of initial rewarming rates are essential to prevent devitrification and quantifying thermal energy dissipation. Thus, prior to measuring nanowarming rates, and assessing sensitivity in electromagnetic environment, temperature measurements of TCs, FO and Cryo FO were compared at room temperature, on ice, and water boiling point. The following present experimental sets of sensors assessment.
3.4.4.1 TC_T

Three temperature sensors: Cryo FO, FO and TC_T were examined upon exposure to AMF (37.5 kA/m and 344 kHz) independently using six samples. Three were DIW, and the remaining three were suspensions of IO MNP at 1 mg of Fe per ml as illustrated in Fig. (3-6). Each sample was 300 µl. The objective of this study was to test each sensor separately in each sample and investigate initial response in an electromagnetic environment and any potential form of induced heating when using TC_T. This is by both comparing results to both types of FO probes in IO MNP and DIW under exposure of AMF.

![Image of IO MNP samples](image_url)

Figure 3-6. IO MNP samples.

3.4.4.2 Soldered TC_T

In an attempt to reduce electromagnetic interferences, two wires of TC_T were soldered and soldered taped, respectively. Thus, in this study, three types of TC_T were compared that varied in the metallic conductive portion of the probe as shown in Fig. (3-7) by using DIW samples upon exposure to AMF (37.5 kA/m and 344 kHz). Soldering was done at the UF chemical engineering shop. Soldering was attempted to enhance bonding at the measurement junction, and taping the soldered part was motivated by the idea of creating an insulation layer. Thus, two wires were soldered once TC_T was made and twisted, one of which was taped.
Figure 3-7. Modified TC_T including normal, taped soldered and soldered.

3.4.4.3 TC_T and TC_E

Thermocouple Type E was examined here. Two tests were performed in electromagnetic environment with and without TC_T in a 300 µl DIW. Thus, TC_E sensitivity to AMF was tested in the presence and absence of TC_T in the sample. To illustrate, the first test was TC_E and FO in one sample. Another, TC_E, TC_T and FO were placed in another sample. Exposure to AMF of 37.5 kA/m and 344 kHz took place. The objective was to examine the reliability of TC_E in an AMF and compare measurements to TC_T and both types of FO.

3.4.4.4 TC_T, TC_T and ready-made TC_T kits

The duality effect of two types of TCs was further investigated here among different combinations of TC_E and TC_T as well as ready-made TC_T kits. The advantage TC_T kits is to eliminate variations associated with the making process of TCs while relying on wire bonding created by the manufacture. Thus, five tests with different combinations were performed here using 300 µl of DIW under exposure of AMF with an amplitude of 37.5 kA/m and frequency of 344 kHz.

3.5 Nanowarming

The objectives of the following studies were to formulate 8 ml of mVS55 with a concentration of 6 mg Fe per ml. Preparation of mVS55 was slightly different than in Section
3.2.2. This difference was with respect to step at which particles were added to the mixture. As opposed to concentrating particles than adding them to the prepared mixture of CPA, particles were concentrated in DIW at the volume added to the mixture. Another difference happened to be the molecular weight of PEG polymer coating IO MNP. In here, PEG-5000 was used.

Another objective is to estimate initial rewarming rate at -196 °C and to calculate and compare values obtained to the ones in recent published studies [35]. A third objective was to compare initial rewarming rate of both mVS55 and VS55 at rewarming modalities of room temperature and in an applied field. A fourth objective happened to be examining rewarming rate in relation to magnetic field amplitude. Last objective was to assess the stability of the particles suspension with respect to higher iron oxide concentration and higher MW of PEG (5kDa).

In the presented studies, the following remained consistent throughout:

- Field conditions used were the same including using 37.5kA amplitude and 348 kHz.
- Cryo FO temperature probe was used to monitor sample temperature whereas FO used to monitor coil temperature.
CHAPTER 4
RESULTS AND DISCUSSION

4.1 Particle Characterization

Prior to suspending particles in CPA mixtures, magnetic and size characterization of PEG-2000-coated IO MNP were performed via SQUID magnetometer and DLS instruments, respectively DIW.

SQUID magnetometer measures the response of magnetic particles to an applied magnetic field. Magnetization vs field plot was obtained illustrated in Fig. (4-1), which was used to estimate the magnetic diameter to be 11.3 nm. The response of PEG-2000 coated IO MNPs to an applied magnetic field via SQUID measurement confirmed the superparamagnetic nature. Next, DLS was used to estimate hydrodynamic diameter of particles prior to suspending in CPA mixture, which happened to be ~30 nm as shown in Fig. (4-2).

![Figure 4-1. Magnetization curve of PEG-2000 coated iron oxide prior to adding to CPAs using SQUID.](image-url)
Figure 4-2. Size characterization of PEG- 2000 coated iron oxide suspended in DIW prior to adding to CPAs using DLS.

4.2 Colloidal Stability

Viscosity measurements were first obtained followed by stability assessment studies of particles in mCPA suspensions and PBS 1X buffer at both room temperature and pre-vitrification and post nanowarming.

4.2.1 Viscosity Measurements

Viscosity measurements were obtained for formulated VS55, VS83, DGPE and PBS 1X. Plot of viscosity values confirmed Newtonian flow behavior illustrated in Fig. (4-3). Furthermore, using conversion factor of 1000 \( \frac{c\text{Poise}}{Pa\cdot s} \), final viscosity values used in DLS measurements are summarized in Table 4-1.
Figure 4-3. Rheology measurements of CPA formulations characterizing viscosity of CPAs solutions using Rheometer at two cycles of increasing and decreasing shear rates.

Table 4-1. A summary of viscosity values used in DLS measurements.

<table>
<thead>
<tr>
<th>CPA</th>
<th>Viscosity [cPoise]</th>
</tr>
</thead>
<tbody>
<tr>
<td>VS55</td>
<td>2.89</td>
</tr>
<tr>
<td>VS83</td>
<td>4.60</td>
</tr>
<tr>
<td>DGPE</td>
<td>3.32</td>
</tr>
<tr>
<td>PBS 1X</td>
<td>0.96</td>
</tr>
</tbody>
</table>

4.2.2 Colloidal Stability at Room Temperature

Hydrodynamic diameter size distribution of PEG-coated IO MNP was monitored at different time points in four suspensions. Colloidal stability refers for the ability of a dispersion to resist aggregation. Mean diameter of particles was ~ 30 nm in DIW prior formulation of mCPA mixtures. As provided in Table 4-2, a relatively similar particle distribution through all time points was observed in mVS55 and mVS83 where average of mean diameter was ~50 nm and ~ 30 nm, respectively. Nonetheless, in mDGPE suspension, mean diameter was approximately more than ~ 300 nm on day 14 which is 6 times more than the initial diameter on
day 1, ~ 50 nm. Thus, particles suspended in DGPE showed agglomeration. This may be attributed to steric and van der Waals repulsion and attraction forces between PEG coating on the particles and hydroxyl groups in glycerol, which is one of the components in DGPE mixture. Aggregation may lead to adverse effects in vasculature leading to non-uniform heating [78]. Thus, this will ultimately lead to thermal stresses combating the objective to using IO MNP of achieving uniform heating. To illustrate, when perfusing organs with mCPAs, occurrence of aggregation may cause clot formation in capillaries leading to difficulty in removing mCPA. Thus, these studies showed potential of two mCPA solutions including VS55 and VS83 while eliminating usage of DGPE as shown in Fig. (4-4). Volume-weighted distributions of each mVS are provided in Appendix A.2.

Table 4-2. Mean hydrodynamic diameter of particles suspended in CPAs and obtained by DLS over two weeks period.

<table>
<thead>
<tr>
<th></th>
<th>PBS1X</th>
<th>VS55</th>
<th>VS83</th>
<th>DGPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter [nm] ± σ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>42.7 ± 2.6</td>
<td>53.4 ± 4.1</td>
<td>30.8 ± 2.4</td>
<td>55.5 ± 4.3</td>
</tr>
<tr>
<td>Day 2</td>
<td>55.1 ± 3.8</td>
<td>52.6 ± 3.2</td>
<td>20.0 ± 1.5</td>
<td>37.4 ± 2.2</td>
</tr>
<tr>
<td>Day 6</td>
<td>41.1 ± 2.5</td>
<td>38.0 ± 2.1</td>
<td>38.9 ± 2.6</td>
<td>40.8 ± 2.6</td>
</tr>
<tr>
<td>Day 14</td>
<td>46.1 ± 3.1</td>
<td>53.0 ± 2.8</td>
<td>34.9 ± 1.9</td>
<td>338.8 ± 50.8</td>
</tr>
</tbody>
</table>
Figure 4-4. Stability of PEG-200 coated IO MNP suspended in CPAs was assessed using DLS at different time points over two weeks showing no aggregation and colloidal stability in all solution except in DGPE.

4.2.3 Pre-vitrification and Post Nanowarming

Stability of IO MNP was also studied by comparing size of particles pre-vitrification and post nanowarming. This was by placing samples in LN$_2$ for approximately 1 week followed by exposure to an AMF. Table 4-3 summarizes mean hydrodynamic of particles in all four mixtures concluding no significant change in the measured hydrodynamic diameter of the particles as shown in Fig. (4-5) as diameter remained in relatively similar distributions as studies performed at RT. Volume-weighted distributions are provided in Appendix A.2.

Table 4-3. Mean hydrodynamic diameter of particles in CPAs obtained by DLS pre-vitrification and post nanowarming.

<table>
<thead>
<tr>
<th></th>
<th>PBS1X</th>
<th>VS55</th>
<th>VS83</th>
<th>DGPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter [nm] ± σ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre- Vitrification</td>
<td>41.1 ± 2.5</td>
<td>38.0 ± 2.1</td>
<td>38.9 ± 2.6</td>
<td>40.8 ± 2.6</td>
</tr>
<tr>
<td>Post nanowarming</td>
<td>48.3 ± 3.0</td>
<td>27.7 ± 2.4</td>
<td>33.5 ± 1.8</td>
<td>34.9 ± 3.0</td>
</tr>
</tbody>
</table>
4.3 Temperature Sensors

Two types of temperature sensors: thermocouple and fiber optics were evaluated and compared in electromagnetic environment. TCs are more sensitive to AMF interferences, which may be attributed to [2]:

- Induce self-heating in the conducting part
- Induce voltage in the thermocouple circuit
- Galvanic coupling between the heated part and the circuit

However, one study demonstrated the impact of size and geometry on the conductive segments of TCs played a role in almost negligible AMF effects [3]. Results of this published study motivated testing and evaluating TCs sensitivity by using similar size of 40 gauge thermocouples. Another motivation to evaluating TCs is the ability to use many channels that may allow using as many TCs to examining different points within the sample at the same time.
4.3.1 TC_T

Three temperature sensors: Cryo FO, FO and TC_T were examined upon exposure to AMF (37.5 kA/m and 233 kHz). Six samples were used: three were DIW, and the remaining three were suspensions of IO MNP at 1 mg of Fe per ml. In DIW samples, initial offset heating rate is \(~30^\circ C/\text{min}\) as coil current was turned on followed by steady temperature rise of \(0.66^\circ C/\text{min}\) using TC_T sensor; the latter is similar to both FO and Cryo FO which is shown in Fig. (4-6B). On the other hand, in IO MNP samples, there is an initial increase of \(~35^\circ C/\text{min}\) as illustrated in Fig. (4-6A) that was followed by steady rate rise of \(3^\circ C/\text{min}\) that is similar to both FO and Cryo FO. To conclude, in both studies, there is a offset temperature rise once coil current was turned on followed by steady rate rise at a similar performance as FO and Cryo FO.

Figure 4-6. Temperature vs time of three temperature sensors: TC_T, FO and cryo FO upon exposure to AMF. (A) IO MNP samples. (B) DIW samples.
Figure 4-6 Continued.

### 4.3.2 Soldered TC_T

An attempt to reduce artifact of induced-heating at the moment of coil current commencement, the conducting segments of two TC_T wires were soldered and soldered taped, respectively as shown in Fig. (3-7). Thus, in this study, three types of TC_T were compared while varying the metallic conductive portion of the probe. This was compared to FO and Cryo FO measurements. Initial offset was very high ~ 2000°C/min and ~40°C/min using taped soldered and soldered, respectively. Therefore, this eliminated the use of soldered and soldered taped in the remaining experiments.
4.3.3 TC_T and TC_E

TC_E was made in a similar manner to TC_T. Two tests were performed. First, all sensors: TC_T, TC_E, and FO were placed in the same DIW sample then subjected to AMF of
37.5 kA/m and 344 kHz. A second test was testing TC-E separately without TC_T and subjected to same conditions of AMF.

In the first test, there was no offset of temperature rise in TC_E temperature plot illustrated in Fig. (4-8) while an appreciable offset was apparent in TC_E in the second test as shown in Fig. (4-9). Estimating temperature gradients once coil current is turned on in both TC_T and TC_E from is 30°C min and 0.66°C/ min, respectively illustrated in Fig. (4-8).

Nonetheless, in the second test, when examining TC_E separately without TC_T in the sample, offset was estimated to be approximately 400°C/ min which is more than 10-fold increase compared to TC_T shown in Fig. (4-6B). Yet, an interesting observation illustrated a suppression effect when TC_T and TC_E are placed in the same sample. The suppression effect on the offset in TC_E in the presence of TC_T also illustrated a steady temperature illustrated in FO illustrated in Fig. (4-8). Furthermore, there was no change in TC_T sensitivity to AMF when placed with TC_E as shown in Fig. (4-8) since initial temperature rise of 30°C/ min was also demonstrated when TC_T was placed separately in DIW sample provided in Fig. (4-6B).

Figure 4-8. Temperature vs Time plot of Three temperature sensors: TC_T, TC_E and FO in DIW sample.
4.3.4 TC_T, TC_E and Ready-made TC_T kits

The objectives of the following studies included comparing bonding of the conductive portions of both TC_T and TC_kit, and to further investigate the suppression effect when pairing two dissimilar types of TCs in the same sample. Each of the following plots represents one pairing of TC sensors placed in DIW sample while being subjected to AMF. Samples’ sizes and applied field amplitude and frequency remained the same.

Types of TCs examined here included: TC_T, TC_kit and TC_E. Both TC_T and TC_kit is composed of same metal components while they differ in their bonding as TC_T was made in the lab and TC_kit was a ready-made by the manufacturer. Results demonstrated a noticeable offset of temperature increase upon turning current on as shown in Fig. (4-10) and Fig. (4-11). Although induced heating is shown to be higher in TC_T/TC_T pair illustrated in Fig. (4-10) when compared to TC_T/TC_kit pair shown in Fig. (4-11) despite being composed of same metal components. Since TC_T and TC_kit differ in the bonding of the conductive components, this highlights the role it is inducing artifact.

Figure 4-9. Temperature vs Time plot of TC_E in DIW upon exposure to AMF.

![Temperature vs Time plot of TC_E in DIW upon exposure to AMF.](image)
In cases of pairing dissimilar types of TCs, a comparable observation of suppression effect is noted in both pairing: in TC_T/ TC_E and TC_kit/ TC_E as shown in Fig. (4-13) and Fig. (4-14), respectively illustrating significant decrease in offset temperature. In fact, when in TC_E/ TC_T pair, Fig. (4-13) shows a steady temperature rise of 8°C/ min in initial TC_E. This is reduced to 6°C/ min in TC_E when coupled with TC_kit as illustrated in Fig. (4-12). In addition, temperature rise in TC_T seems to be significantly higher than TC_kit, which may emphasize the role of bonding in induced-heating.

Last pairing was two probes of TC_kit pairings. As illustrated in Fig. (4-14), an initial increase in temperature was estimated at 35°C/ min and 15°C/ min. Noting an approximately twice as heating in one sensor versus the other might suggest continuous chain temperature rise. To illustrate, induced heating TC_kit1 increases temperature in TC_kit2 and vice versa.

The preceding set of experiments showed inconsistency in obtaining reliable measurements when using TCs in electromagnetic environment as it may lead to overestimation of initial rewarming rates. Therefore, for the remaining studies of nanowarming, Cryo FO was used to monitor mVS55 temperature measurements, and FO was used to monitor the temperature of coil.

![Temperature vs Time plot](image)

Figure 4-10. Temperature vs Time plot of TC_kit and TC_T1 pairing when subjected to AMF.
Figure 4-11. Temperature vs Time plot of TC_T1 and TC_T2 pairing when subjected to AMF.

Figure 4-12. Temperature vs Time plot of TC_kit and TC_E pairing when subjected to AMF.

Figure 4-13. Temperature vs Time plot of TC_E and TC_T1 pairing when subjected to AMF.
4.4 Nanowarming

In the following studies, heating rates were measured and monitored using both FO and Cryo FO. A sample of 8 ml of magnetic VS55 with 6 mg Fe/ ml was prepared then vitrified by immersion in LN$_2$ followed by rewarming through subjecting samples to AMF of 37.5 kA/ m and 348 kHz. Cryo FO was placed in the sample to monitor temperature and ensure sample had reached -196 °C. Also, a sample of 8 ml of VS55 without particles was also prepared then vitrified. Cooling rates of mVS55 and VS55 were comparable and were measured to be ~170°C/min as illustrated in Fig. (4-15A). Also, initial rewarming rate of mVS55 subjected to AMF was measured to be 203.7°C/min. The obtained heating rate was also compared to rewarming without field by rewarming at RT. It was also compared to VS55 rewarming without particles of which VS55 was vitrified and then allowed to rewarm by subjecting to AMF as illustrated in Fig. (4-15B). Results are summarized in Table 4- 4. Furthermore, energy dissipation rate, indicated by SAR, was calculated using obtained initial heating rates. Physical properties of VS55 including both heat capacity and density of 2.033 kJ/ kg K and 1060 kg/ m$^3$, respectively were used in Eq. 3-7 to calculate SAR [24]. To rewarm VS55 without
devitrification, an initial rewarming should be 200°C/ min below $T_g$ [4]. Thus, we were able to achieve rapid initial rewarming rate of 203.7°C/min. Energy dissipation rate of the particles, quantified as SAR, was calculated and the result showed ~ 1210 W/g$_{Fe}$. This is significantly higher than current published work of nanowarming of fibroblasts and vessel segments in which they obtained 160 W/g$_{Fe}$ while using commercially- obtained particles at higher concentrations [35].

Next, examining the potential in adjusting heating rates by varying amplitudes of the applied magnetic field was demonstrated in Fig. (4-16). As predicted, at higher AMF amplitudes, greater energy was released and faster initial rewarming rate were measured starting at a temperature of -196.5°C. Tuning amplitude and frequency of AMF offers great potential in regulating heating rates when translating this concept to a biological specimen as it allows monitoring of temperature within the tissue and so ensuring even heating within the organ. Initial rewarming rates as well as corresponding SAR values are summarized in Table 4-5.
Figure 4-15. (A) Full temperature profile including cooling and rewarming of mVS55 in an AMF and VS55 at RT. (B) Nanowarming of mVS55 in an AMF (37.5 kA/m and 348 kHz) compared to mVS55 at RT with no field (No AMF) and VS55 without IO MNP.

<table>
<thead>
<tr>
<th>AMF</th>
<th>mVS55 [°C/min]</th>
<th>VS55 [°C/min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMF</td>
<td>203.7</td>
<td>26.1</td>
</tr>
<tr>
<td>No AMF</td>
<td>47.7</td>
<td>10.3</td>
</tr>
</tbody>
</table>
Figure 4-16. Control of heating rates of mVS55 through varying AMF amplitudes

Table 4-5. Initial rewarming rates and SAR values of mVS55 upon exposure to various AMF amplitudes

<table>
<thead>
<tr>
<th>AMF Amplitude [kA/m]</th>
<th>37.5</th>
<th>28.1</th>
<th>18.8</th>
<th>9.4</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta T/\Delta t$ [°C/min]</td>
<td>203.7</td>
<td>164.9</td>
<td>100.7</td>
<td>44.0</td>
<td>47.7</td>
</tr>
<tr>
<td>SAR [W/ g Fe]</td>
<td>1219</td>
<td>987</td>
<td>603</td>
<td>263</td>
<td>286</td>
</tr>
</tbody>
</table>

4.5 Stability Check

Final stability assessment was performed since higher particles concentration and higher MW of PEG was used. Particles remained colloidally stable as volume-weight size distributions shown in Fig. (4-17) remained within acceptable range showing no aggregation when comparing particles prior to vitrification right after formulation of mVS55 and post multiple vitrification processes.
Figure 4-17. Pre-vitrification and post rewarming size characterization of PEG-5000 coated IO MNP assessed using DLS
Cryopreservation by vitrification has great promise in extending shelf-life of biologics, and potentially revolutionizing biobanking of tissues and organs. Despite advances in successfully vitrifying macroscopic tissue, it has been shown challenging to successfully vitrify multicellular tissues and organs. This is partially related to the current rewarming modalities that result in devitrification and non-uniform heating. Nanowarming is a novel approach that offers fast controllable heating in volumetric fashion such that biocompatible iron oxide nanoparticles are added to CPAs formulating magnetic CPAs (mCPAs). Once subjected to an alternating magnetic field (AMF), energy is released from particles by magnetic moments of Neel and Brownian relaxations.

Since this is a nascent research area in our lab, preliminary studies of stability were necessary as well proof-of-concept of nanowarming. Colloidal stability studies demonstrated excellent colloidal stability of IO MNP against aggregation in two formulated CPAs: VS55 and VS83. This may be attributed to the dense brushes of poly(ethylene glycol) PEG polymer coating on the particles. Nonetheless, DGPE showed aggregation formation as mean diameter increased significantly over time. Furthermore, particles were stable prior vitrification and post nanowarming.

Investigating reliable temperature sensors to measuring initial heating rates at LN$_2$ by comparing electronic-based thermocouples to optical-based fiber optics showed that despite using appropriate geometry of 40 gauge in thermocouples as recommended by published work [3], TCs are sensitive to AMF interferences demonstrated by induced heating and initial temperature rise upon initiating current. Thus, fiber optics have been proven to be more reliable as they are immune to electromagnetic interferences and were used in nanowarming studies.
It was reported that an initial rewarming rate of 200°C/ min is needed below $T_g$ to prevent devitrification in VS55 such that $T_g$ is -123°C [4, 24]. Thus, this concludes that there was no devitrification in prepared samples since initial rewarming rate was 203.7°C/min. Furthermore, quantifying thermal dissipation by the particles was quantified using SAR resulting in 1210 W/g$_{Fe}$. This is almost 10- fold higher to what was obtained in another published work [35] that reported 160 W/g$_{Fe}$ while using higher iron concentration than what was used in this thesis. Last, regulating thermal energy dissipation by varying AMF amplitudes was demonstrated.
APPENDIX
ADDITIONAL DATA

A.1 mCPA formulations

<table>
<thead>
<tr>
<th>Component</th>
<th>VS55 [g/l]</th>
<th>VS83 [g/l]</th>
<th>DGPE [mg/mg]</th>
<th>Density [g/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>242.14</td>
<td>363.21</td>
<td>0.18</td>
<td>1.10</td>
</tr>
<tr>
<td>PG</td>
<td>168.38</td>
<td>252.57</td>
<td>0.058</td>
<td>1.04</td>
</tr>
<tr>
<td>Formamide</td>
<td>139.56</td>
<td>209.34</td>
<td>-</td>
<td>1.13</td>
</tr>
<tr>
<td>EG</td>
<td>-</td>
<td>-</td>
<td>0.095</td>
<td>1.11</td>
</tr>
<tr>
<td>Glycerol</td>
<td>-</td>
<td>-</td>
<td>0.141</td>
<td>1.12</td>
</tr>
<tr>
<td>HEPES</td>
<td>2.4</td>
<td>2.4</td>
<td>-</td>
<td>1.04</td>
</tr>
<tr>
<td>PBS 1X</td>
<td>20% v/v</td>
<td>20% v/v</td>
<td>To complete</td>
<td>1.00</td>
</tr>
<tr>
<td>DIW</td>
<td>To complete</td>
<td>To complete</td>
<td>-</td>
<td>1.00</td>
</tr>
</tbody>
</table>

A.2 Colloidal Stability

<table>
<thead>
<tr>
<th></th>
<th>Room Temperature</th>
<th>Pre-vitrification and post nanowarming</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS 1X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 14</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Graph of Volume Weighted Distribution](image1)

![Graph of Volume Weighted Distribution](image2)
LIST OF REFERENCES


[66] A. Ardehali, 1. While millions and millions of lives have been saved, organ transplantation still faces massive problems after 50 years; organ preservation is a big part of the solution, Cryobiology. 71 (2015) 164–165.


BIOGRAPHICAL SKETCH

Eman Shreteh completed her undergraduate in the University of Florida, Gainesville, Florida, where she received her Bachelor of Science in Chemical Engineering and Bachelor of Arts in Mathematics in December 2015. She continued pursuing her master’s degree by joining the J. Crayton Pruitt Family Department of Biomedical Engineering at the University of Florida in August 2016.