Meta-Review

Genetic Storage-Expansive Dualistic Strategies for Engineering Cryo-resistant Bioartificial Tissues – A Meta-Review

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Abstract

Cryogenic bioartificial tissue preservation remains a critical challenge, primarily due to the reliance on cytotoxic cryoprotectants for ice crystal inhibition. Recent advances in synthetic biology offer promising alternatives by enabling the endogenous intracellular cryoprotection. This review explores the potential of genetically-encoded cryoresistance, drawing insights from natural systems such as antifreeze proteins (AFPs), heat-shock proteins (HSPs), and cold-adaptive pathways. It reviews key molecular features—solvation thermodynamics, hydrogen-bonding networks, and membrane-stabilizing motifs—used to inhibit ice formation and mitigate damage in engineered tissues. Computational and experimental studies indicate that genetic modifications promoting the expression of cryoactive proteins can enhance cell viability across thermal transitions, from normothermic culture to cryogenic storage. Furthermore, we discuss how synthetic gene circuits and pathways can optimize cryoresistance while minimizing metabolic and structural disintegration. By integrating biophysical modelling with genetic design principles, this review highlights strategies for developing next-generation bioartificial tissues capable of even, ubiquitous, scalable and endogenous cold adaptation. Finally, we assess translational challenges, including scalability, safety, and regulatory considerations, while outlining future directions for clinical implementation. This genetic engineering paradigm holds significant potential to overcome the limitations of traditional cryopreservation, advancing the field towards more robust and clinically viable tissue-based therapies.

Keywords: cryopreservation, molecular engineering, genetic engineering, osmolytes, antifreeze glycoproteins, translocation free energy, heat shock proteins, cold-adaptive pathways

1. Introduction

"Thermic" resistance is a widespread natural phenomenon, and its dual capacity to endure both cold and heat stresses offers valuable insights for biotechnological storage-expansion protocols. Bioartificial constructs have to endure temperatures as low as -196 degrees Celsius and as high as 37 degrees Celsius not to mention changing levels of oxygen tension, changing biomechanical substrate milieus, mechanical agitation and frequently, enzymatic digestion. In nature, examples of thermic adaptations abound. For instance, insects[1], fish[2], reptiles[3] and amphibians[3] deploy distinct protective strategies depending on the thermal challenge: they upregulate heat-shock proteins and adjust cytosolic & membrane fluidity[4] in response to heat stress, while synthesizing antifreeze proteins and accumulating endogenous biomolecular cryoprotectants and osmoprotectants[3,5] across biophysically relevant thermal regimes —they demonstrate their ability to toggle specialized stress responses to survive thermal extremes. On another distinct phylogenetic branch, Atlantic fishes[6] have co-opted ancient genes—originally involved in basic cellular homeostasis —to chaperone proteins under ice-generating conditions, revealing a latent molecular toolkit for cold tolerance. Intriguingly, mammalian cells also activate endogenous stress pathways when challenged by temperature extremes, hinting at innate cryoresistance mechanisms.[7] By bridging these natural adaptations—from invertebrates to fish to mammals—one can strategically harness and integrate these mechanisms to enhance cell viability, scalability, and overall bioartificial tissue robustness during storage and expansion.[8]

2. Adaptations to the biomedical need

Scalable bioartificial tissue production is essential for regenerative biology to meet the full gamut of biomedical demand spanning acute and chronic care in a timely fashion. Bioartificial tissues can be used extracorporeally and intracorporeally.[9] When used as a temporary measure, they can act as life support systems deployable at all stages from field rapid response to life-saving surgery.[9] However, scalable bioartificial tissue procedures must overcome size limitations to achieve the desired utility in a real-world context. Procedures that rely on synthetic and/or exogenous cryoprotectants and osmoprotectants tend to have size limitations imposed by diffusion limits which scale the diffusion time proportionally to the square of the biological construct's characteristic size and inversely proportional to the diffusivity.[10] The presence of extracellular material will impact both terms negatively resulting in greater size constraints.[10] Endogenous systems could be a resolution to this problem since the protectant molecules would be present in situ therefore frog-jumping the diffusion challenge.[8] Therefore, having endogenous systems, much like the cold-adapted insect, amphibian and fish, can enable a continuum of storage solutions from hyperthermic, normothermic, hypothermic to cryothermic storage.

3. Novel developments in cryopreservation

Given the challenges observed in using synthetic penetrating cryoprotectants such as dimethyl sulfoxide (DMSO)[11–13], there are efforts to broaden cryoprotectants into benign, biocompatible equivalents[12]. This section will evaluate these mechanisms broadly but within the central goals of endogenous intracellular production of such cryoprotectants to overcome the cell membrane barrier.

3.1. Beyond Membrane viability testing

Research into novel mechanisms of cryoprotection focuses not on the idealistic complete elimination of freezing damage, but on limiting ice crystallization/recrystallization damage below critical thresholds and later enabling supercharged cellular recovery processes post-thaw. Recognizing that cells passing simple viability tests might still fail at recovery[14], comprehensive assessment requires functional/phenotype tests (e.g., albumin production, pluripotency[11]) and evaluation of cell fate processes like proliferation and migration[11]. Cell viability testing that relies on membrane intactness could initially appear high due to biased sampling or delayed membrane disintegration[15]. On the other hand, it may be that cell layers with partial intactness might nevertheless recover membrane integrity under [16]. A strange midpoint may be cells that display functional membrane integrity tests but fail to fulfil all cell phenotypic characteristics due to a lack of underlying support mechanisms[11,16].

3.2. Cold-Hardening

Due to these complexities of determining holistic cell viability, sample preparation procedures are crucial, including cold-hardening[17], activating house-keeping/degradation protein-cycling mechanisms[18], and employing chaperones & co-chaperones (HSPs) for maintaining a proper fold-character in the functional proteome[18] of the cells pre-freeze and post-thaw. Strategies like cold -hardening[17], which expose cells to brief spells of hypothermic culture, and cell serum starvation, involving a reduction of the serum-derived factors that spur growth and division, will together induce metabolic hibernation by encouraging cells into the protective genetic state, where condensed, transcriptionally suppressed[19], genetic material is less damage-prone[3,20]. Such strategies will also engage degradation mechanisms necessary to avoid potential auto-disruptive misfolded proteins from initiating a cascade of unwanted protein aggregations[3].

3.3. AFP-driven protection Mechanisms

3.3.1. Hydrophilic-Kinetic Effects

Besides activated cellular housekeeping, some mechanisms may involve the introduction of ice-disrupting molecules such as AFPs. AFPs, particularly through their hydrophilic interfaces, interact with the surrounding water molecules in a way that disrupts the arrangement of an ordered, ice-like structure creating zones of disordered stacks of water molecules[21]. This "nucleation-blocking effect" elevates the energy barrier[22] for water molecules to self-assemble into a critical ice nucleus, thereby kinetically delaying or inhibiting[22] the initial formation of ice seeds in both intracellular and extracellular environments. This is a direct consequence of the hydrophilic interface's distinct surface chemistry[21,22], which prevents the templating of water into an ice lattice.

3.3.2. Hydrophobic Adsorptive interface

This adsorption-inhibition mechanism physically blocks the advancement of the ice front, forcing growth into highly curved, energetically unfavourable geometries[22]. This effectively "pins" the ice crystals, preventing their uncontrolled expansion and subsequent mechanical damage to cellular structures. This effect involves specific interfacial interactions[21,22] and the disruption of the regular hydrogen bond network across the ice-AFP-water interface[21].

3.3.3. Inhibition of Ostwald Ripening

AFPs actively suppress the Ostwald ripening process, where larger ice crystals grow at the expense of smaller ones. By binding to ice crystal surfaces regardless of surface area-to-volume ratio, AFPs prevent the migration of water molecules from smaller, more highly curved crystals to larger ones[23], thereby shifting the first-moment population parameters such as crystal diameters to the left resulting in less damaging ice crystals[24]. This is crucial for maintaining cell viability. Overall, the twin effects result in a synergistic broadening of the thermal hysteresis[23] gap even when the bulk water might contain potential nucleation particles. In this respect, AFPS guide the structural arrangements of water molecules around membranes, inclusion bodies and other cellular components in highly stable hydration shells.

3.3.4. Synergy between intracellular AFPs & extra-cellular Polyols

During congelation procedures, agents like AFPs exert protection through multiple effects: nucleation-blocking effects will disrupt the seed particle/ice nucleus interactions with the surrounding water shell through adsorption-inhibition, facial, second-order amphiphilic effects resulting in kinetically depressed nucleation, intracellular growth-blocking via interfacial effects and hydrogen bond-network disruption, ice recrystallization inhibition, hydrophilic-lipophilic balance variations isolating hydration shells, and deepening the thermal hysteresis in the bulk aqueous medium[23,24]. Extracellular polyols can further inhibit extracellular ice crystal growth through viscosity-driven vitrifying effects[25], freezing point depression[24,25] and replacing lost structural water at membrane interfaces[25]. Intracellularly, polyols may help to accentuate the water ex-osmosis should slow-cooling rates be adopted[26,27] to allow the joint synergy between lower solubilities of extracellular osmolytes drawing out water from the intracellular compartments[26,27] and the favourable hydrophilic interactions proffered by the branching polyol network.

3.3.5. Genetic Adjunct

It is clear that these biomimetic mechanisms for the cryoprotection are multiform and novel biocompatible introductions into the cellular environment. Additionally, unlike synthetic cryoprotectants such as dimethyl sulfoxide, these biocompatible alternatives are effective at low concentrations and are, by design, non-cytotoxic at elevated temperatures[28]. In the post-thaw environment, these biocompatible CPAs are also unlikely to cause sudden phenotypic changes leading to a loss of function at all biological architectures given the pre-existing genetic circuitry that enables internal regulation via gene silencing[28]. Hence, a single technological platform to deliver these benefits may be feasible. Genetic transfection could enable the cells themselves to become the "cryoprotectant factory" at mild pre-loading and cold-hardening stages[29]. Such a platform may extend beyond enabling the endogenous production of wide-ranging components including chaperones and AFPs. Transfection may enable the intracellular production of osmotic synthases thereby allowing cells and bioartificial constructs so-designed to endogenously attain sufficient intracellular cryoprotective osmolytes like trehalose or proline[30]. Additional protective proteins may include Late Embryogenesis Abundant (LEA) Proteins. These are intrinsically disordered hydrophilic proteins, abundant in organisms with a tolerance for extreme conditions including cold and desiccation[31]. Therefore, these LEAs may display cryoprotective[32] and antioxidant properties[32] as colligative conditions become less conducive to normal mammalian cellular metabolism. A novel observed mechanism is their preferential adsorption to the water meniscus and the reduction of unwanted surface-induced protein aggregation[33,34] during freeze-thaw cycles. Such an approach could achieve optimal intracellular concentrations with no risk to the exposure to potentially cytotoxic or post-thaw morphogenic synthetic CPAs. On a biophysical dimension, transfection might even enable the adaptive modulation of the expression and the programmable activity of aquaporins (see ***Figure 1***) to the ultimate fine-point of fine-tuning water trans-osmosis across cell membranes throughout the storage-expansion cycle.

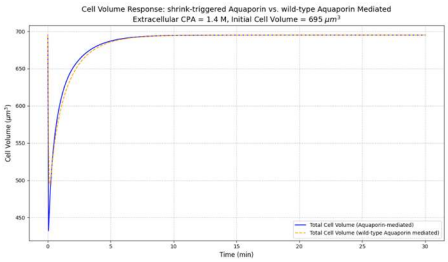

Figure 1. Controllable shrinkage-triggered aquaporins can be useful to prevent extreme deviations as shown by the prevention of the cell volume from dipping below y=490 μm³ (red line). This phenomenon may be inducible via polyol encapsulation. Parameters drawn from mammalian cells[35].

Together, these genetic interventions also overcome the often-intractable challenge of overcoming size-constraints in biopreservation. As sample sizes grow, classical CPA loading approaches that rely on and are constrained by diffusion under Fick's second Law (***Figure 2***) suffer given diffusion is directly proportional to the characteristic distance of the bioconstruct and inversely proportional to the square of the diffusivity of the CPA in the medium-and-construct[10,35].

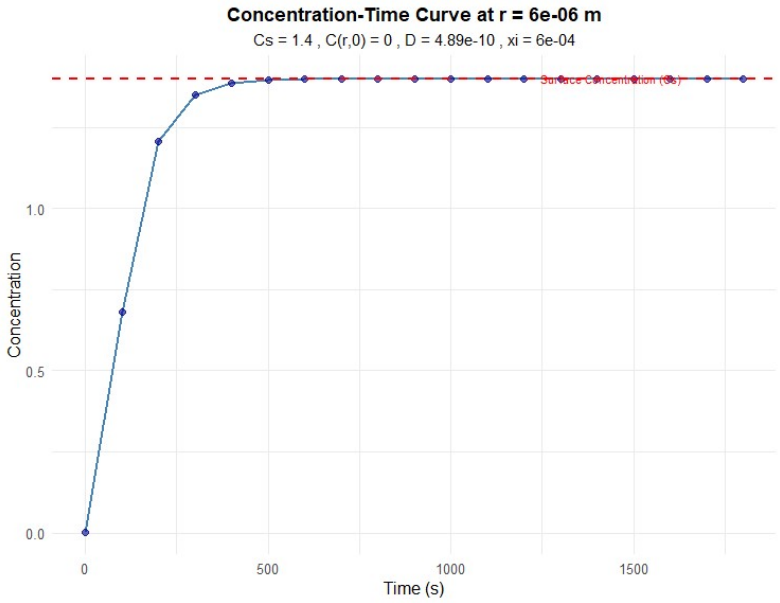

Figure 2. Implementation of Fick's second law to the diffusion of CPA into a 0.6 mm construct showing the requirement for steady-state met at 28% of the total loading time leaving 72% of the loading time for cellular equilibration across the full construct. However, due to DMSO toxicity at room temperature, loading has to be conducted at hypothermic temperatures (4 deg. Celsius). Time in seconds and Concentration in Molar.[36]

A genetically pre-programmable CPA loading method will be in-situ and thusly not constrained in size by design as shown in the ***Figure 3*** whereby the medium and large constructs fail to attain a steady state meaning[10] cells closer to the centre of the spherical construct will tend to suffer from inadequate CPA loading leading to lower survival rates post-thaw. Genetic constructs with endogenous biocompatible[37] CPA production would avoid this problem[8] by rendering the characteristic radius of the construct virtually null i.e., all cells will be like the cells at the surface of the construct with the steady state CPA loading as an initial condition.

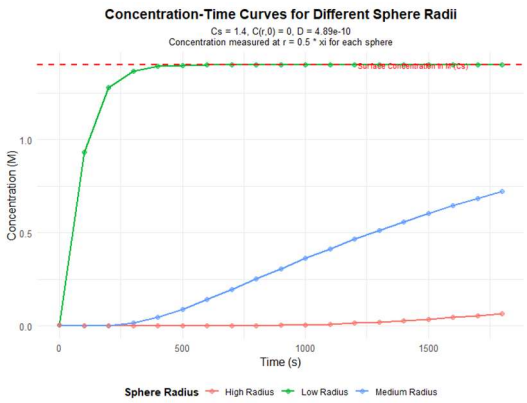

Figure 3. Implementation of Fick's second law to the diffusion of CPA into three spherical constructs of (low) 0.6 mm, (mid) 3 mm and (high) 6 mm radial constructs showing the requirement for steady–state is only met in the small construct.[10]

Advanced thawing procedures move beyond ice-baths, utilizing nanowarming (magnetic/sonar/EF in the VHF range) for uniform heating[37–39]. Critical post-thaw procedures involve inhibiting programmable cell death and restarting the cell cycle. This includes biochemical apoptosis inhibition (e.g., ROCK inhibition)[11], reapplying chaperones & co-chaperones (potentially pre-programmed)[40,41], and biophysical procedures like RGDS encapsulation[11,42,43] for biomechanical induction. Transcellular/cyto-composite induction (e.g., laminins and RGDS-alginates for stem cells) may also be necessary[11,44].

Finally, robust recovery metrics – often overlooked but critical – must assess stemness[11], expansionary capacity[45], and functional/tissue-physiologic[37] performance to truly gauge success in the relevant post-thaw regimes.

3.3.6. Translational challenges

There are critical and often overlooked translational challenges in cryopreservation, particularly when moving beyond simple cell suspensions to more complex systems like bioartificial tissue constructs, organoids, stem cells, hepatocytes, and even whole organisms. Among others, delayed apoptosis[45], spontaneous differentiation, phenotypic drift, immunogenicity[37], and homeostatic disruption[46] – are significant barriers to successful clinical and research translation. There are ways to minimize these effects, some have been explored in the current article, however, the reality exists that these will continue despite best efforts to minimize them. As such, there will continue to be a need to monitor such effects in organismic and latterly clinical studies. For instance, phenotypic testing, among other functional engraftment studies, should be performed for months to years to ensure phenotype stability. Additionally, the potential for immunogenicity might be heightened if stress tolerance factors are released post-implantation[37]. Therefore, bio-artificial tissues and organoids will need a restoration period[37] to monitor potential release of factors such as HSPs. Ultimately, drift-driven oncogenic transformation, or tissue dysplasias in complex systems and other genomic/phenotypic instabilities may necessitate only transient transfection[8] for ethical and safety reasons. Transience by design may help reduce any emergent divergent cell population, leaving behind a durable functional population after replication[8]. By making functional engraftment assays part of the clinical procedures, the kill switch becomes part of the treatment and not a symptomatic knee jerk reaction that returns the patient to the original diseased state. A potential preventative strategy may be to embrace cell encapsulation[10,11] that could allow cryoprotection, bio-mechanical signaling[11], nutrient-CPA exchange[10] but prevent the release of immunogenic factors that were previously cryoprotective.

3.3.7. Delivery Mechanisms

Harnessing endogenous cryoprotection and enhancing cellular resilience are key strategies for advanced cell preservation. One approach involves the genetic transformation of mammalian cells to introduce or upregulate the production of various cryoprotective agents[8]. This can be achieved via electroporation[47] and biologicallyt[8,47,48], delivering genetic material for the expression of stress-tolerance activators such as Antifreeze Proteins[8] (AFPs) and osmoprotectants such as trehalose[47] that are normally impermeable to cell membranes. Furthermore, biomaterial translocation, utilizing physiologically cationic mucoadhesive carriers like chitosan[49] which can among other things, leverage integrin-mediated transport and form stable nanoscale polymer-genetic complexes[49], facilitates the intracellular delivery of these beneficial

molecules whilst enabling potential degradation via endosomic escape. Additionally, inducing tolerance through controlled environmental stimuli, such as mild heat stress for Heat Shock Protein (HSP) production[50], primes cells for increased survival under challenging cryopreservation conditions by reducing proteotoxic stress from aggregated misfolding proteins[41], engaging protein clearance and cycling, inhibiting apoptosis[17], amplifying the generation of antioxidants, accelerating DNA repair and stabilizing cytoskeletal organization which helps preserve membrane structural integrity and intracellular compartmental stability[41].

4. Conclusions

In conclusion, these recent developments signal a meaningful shift in the feasibility and fidelity of safely transformed mammalian cryopreservation via endogenous mechanisms. The successful transfection of genetic cassettes providing endogenous AFPs, osmolytes/osmoprotectant synthases, molecular chaperones and co-chaperones, have demonstrated the ability to enhance post-thaw cell viability, while pre-conditioning strategies such as cold-hardening have shown measurable improvements in preservation outcomes beyond membrane integrity. Advances in encapsulation techniques continue to support structural and functional integrity during freeze–thaw cycles. Notably, cell adhesion to engineered biomaterial substrates—such as RGDS-modified substrates—has emerged as a key strategy for reducing dependence on high concentrations of cryoprotective agents (CPAs), many of which carry meaningful risks of cytotoxicity and phenotypic instability. These substrates also contribute to improved post-thaw phenotypic stability and limit genetic or clonal drift, suggesting that substrate design plays an underappreciated role in preservation biomedical engineering. Finally, initial organ-level studies have begun to show reductions in dysfunction following hypothermic storage, further reinforcing the translational relevance of these combined approaches. Together, these findings point toward a convergent path of molecular, genetic, biomaterials, and process-level innovations that are reshaping the landscape of biopreservation.

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Abbreviations

The following abbreviations are used in this manuscript:

AFPs	Antifreeze Proteins
HSPs	Heat-Shock Proteins
CPA	Cryoprotective Agent/ Cryoprotectant
VHF	Very High Frequency
ROCK	Rho-associated (Protein) Kinases
RGDS	Arginine (R) – Glycine (G) – Aspartic Acid (D) – Serine (S) amino acid sequence

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