Bruno Miguel Soares Guerreiro
Licenciado em Bioquímica

Development of cryoprotectant fluids based on a natural polysaccharide

Dissertação para obtenção do Grau de Mestre em Bioquímica

Orientador: Doutora Maria Filomena Andrade de Freitas,
Senior Researcher, FCT-UNL

Co-orientador: Doutor João Carlos Lima,
Associate Professor with Aggregation, FCT-UNL

Presidente: Prof. Doutor José Ricardo Ramos Franco Tavares
Arguente: Prof. Doutora Maria Alexandra Núncio de Carvalho Ramos Fernandes
Vogal: Prof. Doutora Maria Filomena Andrade de Freitas

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Setembro 2018

Bruno Miguel Soares Guerreiro

Licenciado em Bioquímica

Development of cryoprotectant fluids based on a natural polysaccharide

Dissertação para obtenção do Grau de Mestre em Bioquímica

Orientador: Doutora Maria Filomena Andrade de Freitas

Co-orientador: Doutor João Carlos Lima

Setembro 2018

AGRADECIMENTOS

À Dra. Maria Ascensão Reis, por se comprometer em aceitar-me no grupo e desenvolver este trabalho, sem nunca colocar restrições logísticas ou comerciais. À Dra. Filomena Freitas, pela presença constante, suporte incansável e por me dar a liberdade de seguir uma área que incendeia o meu espírito criativo. Ao Prof. João Carlos Lima, por apoiar as minhas loucuras científicas desde a licenciatura e, uma vez mais, estar sempre aberto a experiências sem igual. Ao Prof. Jorge Silva, por contribuir numa grande parte do meu trabalho com os seus recursos laboratoriais e intelectuais sem qualquer condicionante. Mesmo sem ser meu orientador oficial, não me distinguiu dos seus próprios alunos. À Prof. Madalena Dionísio: sem a sua proatividade, provavelmente nunca teria tido a oportunidade de me licenciar, quanto mais de me formar como Mestre em Bioquímica. Realmente influenciou a minha vida, e isso irei guardar para sempre. Obrigado pelo seu esforço.

À Lígia Coelho, minha companheira de mestrado, sempre juntos e acabámos por parar no mesmo laboratório! À Diana Querido, a quem devo cada dia de felicidade no laboratório, sempre brilhante. À Daniela Fernandes, que por mais longe que estivesses, estavas sempre lá ao meu lado. À Jeniffer Farias, a rapariga sempre de férias que trazia o fogo brasileiro à nossa vida. Nas frustrações, ensaios falhados, sucessos, vida social e privada também, estiveram lá comigo como se de uma família nos tratássemos. Obrigado a todas. À equipa Rijo, nomeadamente aos meus mais próximos: Luís Silva, Rafael Rippel, João Ramos, Diogo Gonçalves. Agradeço todas as vossas tentativas de destronar a minha motivação em resolver os problemas que esta tese me trouxe, regozijando na torrente dos vossos memes. Prometo, esta sim é uma tese a sério. Venham os prémios, a escalada, o surf, o ginásio e os bíceps. Ao Tiago Moreira, Artur Moro, Nuno Basílio e Rita Fernandes pelo vosso apoio nas minhas dúvidas existenciais de fotoquímica e marcações fluorescentes. À Joana Martins, pelas tuas pequenas palavras de encorajamento e motivação que me surpreenderam vezes sem conta. É possível que não tenhas noção o quão moralizador foi o teu interesse pelo meu trabalho, tens um grande caráter. Ao David Peitinho, o meu irmão de armas: discutimos problemas e arranjámos soluções no meio daquelas cantorias de uma hora de boleia para apanhar o comboio. Realmente um grande amigo sem necessidade de manutenção.

Aos meus pais, eles sabem porquê. Passados 23 anos, meia palavra basta.

Por fim, uma mensagem ao eu do futuro: nunca te esqueças das tuas origens.

ACKNOWLEDGEMENTS

CRYO2018 in Madrid this July changed my state of mind forever. In the midst of over 300 people from 35 countries, I travelled alone and for one week, I haven't felt more at home in that conference room. Reuniting with the same faces everyday, it is you – Krishnaa, Nikola, Martin, Daniela, Natalia, Jaume and friends – who I felt most at ease with. Dr. Greg Fahy, a special thank you to you, who treated me with the utmost respect and comfort, made my hard work feel noticed by the best and wasted several hours in my improvement. All your teachings made me realize how lucky I was to be there in the first place. I never thought I would be given so many life lessons in a scientific conference, some from dialog while others subconsciously. Although I felt homesick in Spain, I had the same feeling flying back. Thank you all.

"I was waiting for something extraordinary
to happen, but as the years wasted on,
nothing ever did unless
I caused it."

- Charles Bukowski

Às gerações futuras.

RESUMO

A área da preservação tenta responder ao défice de órgãos enquanto crise mundial ao desenvolver técnicas e formulações crioprotetoras que garantam viabilidade pós-congelação. O FucoPol é um polissacárido rico em fucose produzido pela *Enterobacter* A47 que tem um comportamento pseudoplástico e é estável numa larga gama de temperaturas e ambientes químicos. Este trabalho visou a implementação de FucoPol em formulações crioprotetoras no intuito de simplificar a complexidade das formulações, maximizando a sua função.

FucoPol demonstrou estabilidade química na presença de BSA e da formulação crioprotetora Euro-Collins. Análises termodinâmicas demonstraram que o FucoPol tem capacidade de inibição cristalina comparável à proteína anticongelante tipo I HPLC6, mas é inadequado para vitrificação. Ensaios *in vitro* demonstraram que FucoPol não é citotóxico até 1% m/v, aumenta consistentemente a viabilidade celular pós-descongelação quando adicionado a diferentes meios e é compatível com diferentes linhagens celulares. Esta capacidade crioprotetora aparenta ser proporcional ao conteúdo em fucose. Adicionalmente, FucoPol sinergiza com DMSO em crioproteção enquanto reduz a sua citotoxicidade à temperatura ambiente.

Para abordar o dano metabólico pós-descongelamento, as propriedades antioxidantes do FucoPol foram testadas através de ensaios FRAP. FucoPol neutraliza linearmente espécies oxidativas Fe^{3+}, comportamento comparável ao ácido ascórbico a 0.25% m/v (concentração de trabalho). Adicionalmente, é capaz de neutralizar ROS derivados de H_2O_2 in vitro. A substituição de colóides e impermeantes por FucoPol nas formulações crioprotetoras Euro-Collins, Unisol-CV e Custodiol-HTK demonstrou que a viabilidade mantém-se ou aumenta em alguns casos.

Concluindo, FucoPol é uma molécula multifuncional que beneficia a sobrevivência celular ao fornecer proteção contra estímulos físicos e químicos. Uma vez que otimizar formulações é um processo altamente empírico, um modelo descritivo de implementação de polissacáridos é apresentado para aumentar a eficiência de triagem molecular para aplicações criobiológicas. Por fim, o FucoPol tem potencial na cosmética uma vez que protege células contra danos de radiação UV.

PALAVRAS-CHAVE

FucoPol; criopreservação; exopolissacáridos; fórmula; antioxidante; multifuncional

ABSTRACT

Preservation science tries to answer the worldwide crisis of organ shortage by developing new techniques and cryoprotective formulations to ensure post-storage organ viability. FucoPol is a fucose-rich polysaccharide produced by *Enterobacter* A47 that has a shear-thinning behavior and is stable in a wide range of temperatures and chemical environments. This work envisioned the implementation of FucoPol in cryoprotective fluid formulation with the objective of simplifying formula complexity while maximizing performance.

FucoPol showed chemical stability in the presence of BSA and the cryoprotective formula Euro-Collins. Thermodynamic analysis showed FucoPol has an ice crystallization inhibition behavior similar to the type I antifreeze protein HPLC6, but is not suited for vitrification. *In vitro* assays demonstrated that FucoPol is not cytotoxic up to a concentration of 1% w/v, consistently increases cell post-thaw viability when added to different medium compositions and is compatible with several cell lines. This cryoprotective ability seems to be proportional to its fucose content. Also, FucoPol synergizes with DMSO in crystal avoidance and is able to reduce its cytotoxicity at 37ºC.

To address post-thaw metabolic damage, the antioxidant properties of FucoPol were tested by FRAP assays. FucoPol provides a dose-dependent scavenging of Fe^{3+} oxidative species comparable to ascorbic acid at 0.25% w/v (working concentration) and is able to scavenge H_2O_2-derived ROS *in vitro*. Furthermore, the substitution of colloids and impermeants by FucoPol in the cryoprotective formulas Euro-Collins, Unisol-CV and Custodiol-HTK showed that viability could be maintained or increased in some cases.

Thus, FucoPol is a multifunctional molecule that benefits cell survival by providing protection against both physical and chemical stimuli. Because formula optimization is a highly empirical process, a descriptive model for polysaccharide implementation was designed to increase efficiency in molecular screening for cryobiological applications. Additionally, FucoPol also has potential in cosmetic applications as it protects cells against UV radiation-induced damage.

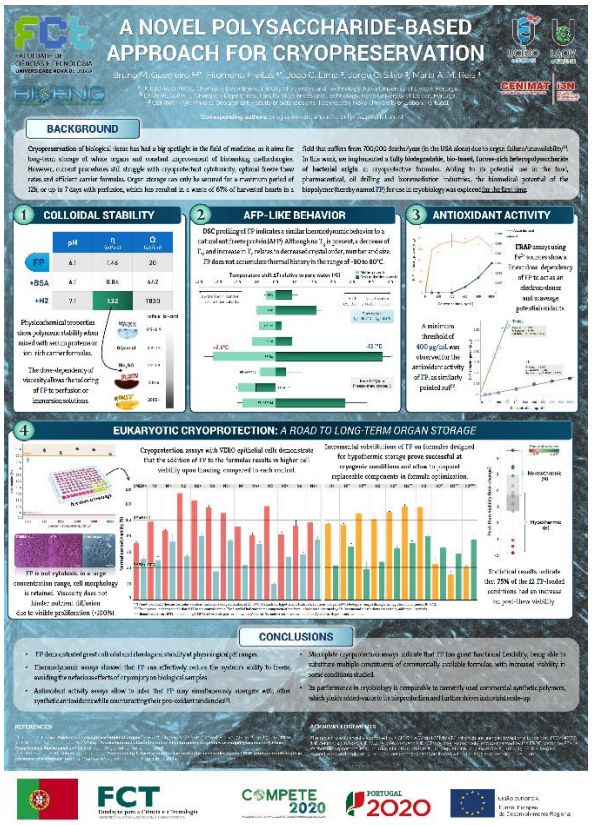
KEYWORDS

FucoPol; cryopreservation; exopolysaccharides; formula; antioxidant; multifunctional

COMMUNICATIONS

This work was divulged twice during the development of this thesis, and both instances contributed to my self-growth and development as a scientist, as well as the welfare of the work itself.

Poster communication



This work was accepted at the 6th Portuguese Young Chemists Meeting held in Setúbal, Portugal.

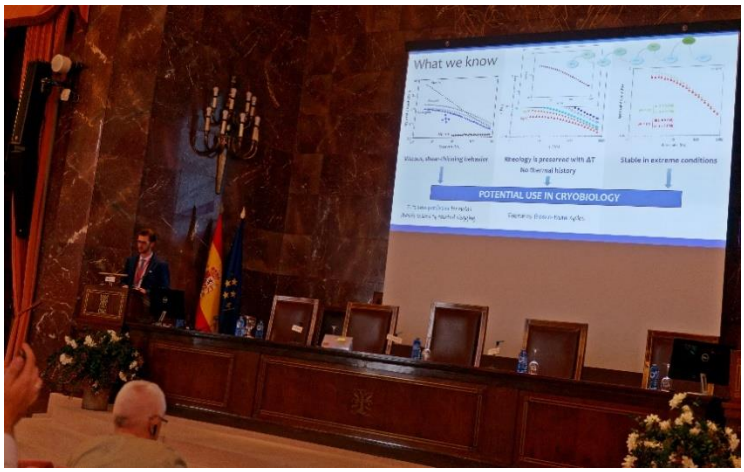
The work was featured as a poster titled:

A novel polysaccharide-based approach for cryopreservation.

Bruno M. Guerreiro,
Filomena Freitas,
João C. Lima,
Jorge C. Silva,
Maria A. M. Reis.

http://6pychem.eventos.chemistry.pt

Oral communication



www.societyforcryobiology.org

This work was accepted at the CRYO2018 International Meeting held in Madrid, Spain by the Society for Cryobiology.

The work was featured as a student presentation titled:
A novel polysaccharide-based approach for cryopreservation,

to which a Student Travel Award was prized and will feature in the December edition of the Cryobiology journal as a conference proceeding.

CONTENT INDEX

FIGURE INDEX

TABLE INDEX

ABBREVIATIONS

AA	*Antifreeze activity*
AFP	*Antifreeze protein*
ATP	*Adenosine triphosphate*
BSA	*Bovine serum albumin*
CFA	*Continuous flow analysis*
CPA	*Cryoprotective agent*
C_p	*Heat capacity at constant pressure*
CTN	*Cryoprotectant toxicity neutralization*
dH_2O	*Distilled water*
DMSO	*Dimethyl sulfoxide*
DSC	*Differential scanning calorimetry*
ECF	*Intracellular fluid-type solution*
EIF	*Extracellular ice formation*
FBS	*Fetal bovine serum*
FP / ✣	*FucoPol*
FRAP	*Ferric reducing antioxidant power*
HC	*Hypothermic continuum*
HPLC	*High-pressure liquid chromatography*
ICF	*Intracellular fluid-type solution*
IIF	*Intracellular ice formation*
IRI	*Ischemia-reperfusion injury*
J_{gr}	*Growth rate*
J_{nucl}	*Nucleation rate*
MDa	*Megadalton (10^6 Da)*
MPT	*Mitochondrial permeability transition*
$O_2^{\cdot-}$	*Superoxide radical*
$OH^{\cdot}$	*Hydroxyl radical*
ROS	*Reactive oxygen species*
T_c	*Crystallization temperature*
T_g	*Glass transition temperature*
TH	*Thermal hysteresis*
T_m	*Melting temperature*

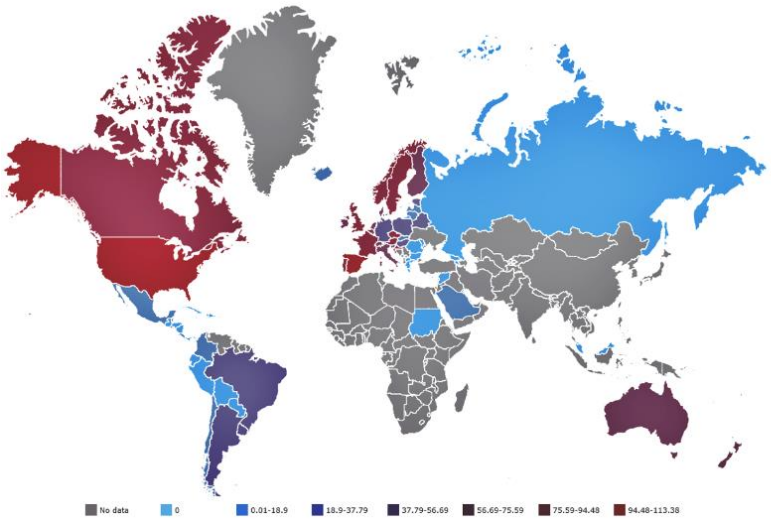
I. BACKGROUND

Preservation science has been a low-key field for quite some decades now, and though research is continuous, the challenges it faces are still often overlooked. The general public is usually unaware of the potential of preserving biological samples for future use. Although it may be enticing to believe that simply freezing a sample might preserve it, the process is really hard to perform without any collateral damage.

1. Preservation: a global health problem

Our inability of efficiently preserving tissues and organs has proved to be very consequential in scientific advances and medical needs. The World Health Organization reports organ shortage to be as much of a global crisis as cancer. In fact, 730,000 deaths/year are attributed to end-stage organ disease, surpassing those of cancer. However, only 10% of patients entitled to a transplantation procedure actually get one[1]. With ever-growing waiting lists, the reality is that there are 10-fold more patients needing a heart transplant than those already waiting[2].

Donor-acceptor compatibility, geographical distribution and low shelf-life are some of the limitations in preservation science. It is estimated that removing such constraints could prevent more than 30% of disease-related deaths[1].



Actual number of utilized deceased organ donors (2017)

Rank	Country	*pmp*
1	Spain	41.08
2	Croatia	31.43
3	Portugal	31.26
4	Belgium	30.53
5	Malta	30
6	USA	29.91
7	France	28.95
8	Italy	24.19
9	Austria	23.68
10	Czech Republic	23.49
45	Russia	3.92

Figure I.1. Global overview on the transplantation rate per million citizens. The issue of organ deficit goes beyond the USA and most of Eurasian/African countries do not possess the means to attend this crisis (2017 census from www.transplant-observatory.org). USA places 6th in donor organ conversion to successful transplants. Portugal has a better conversion rate but only performed 895 organ transplants, given its 10.3 million inhabitants (pmp = absolute value per million population).

If this is the scenario for the United States of America, which is in the forefront of medicine, statistics are even grimmer everywhere else. Africa contains about 16% of the world's population[3] but is only able to perform 0.5% of all required organ transplants (as of 2018)[1], which amounts to 1 in every 200 patients.

Regardless of technology being able or unable to produce fully synthetic organ replacements, long-term preservation of natural harvest is essential, must be preemptively secured and requires perfection of methodology. For every 3 hearts and lungs harvested, a staggering 2 are discarded

and about 26% of donated livers suffer the same fate because they are kept in storage for excessive amounts of time, and when need for transplantation arises, its viability is long lost[4]. Despite exploring different cryoprotectants and even improving patient matching, the knowledge attained still falls short from what clinical practice has always demanded.

1.1. The institutional barrier

To tackle the supply and demand of organ shortage in the 21st century, two main requisites must be fulfilled:

i. Sufficient availability of biological resources to meet population needs;
ii. Means of efficient storage and quick transportation worldwide.

While the first relies greatly on tissue engineering[5], the lack of efficient preservation know-how is a big obstacle hindering any advances in this field. Both fronts are perpetually interconnected and often researchers meet cross-roads that require advances in one field to elucidate the other. Now, while tissue engineering is being widely addressed, the challenge in preservation has been given little attention: the general public seems gravely unaware, funding agencies strike little interest in the subject, and research development has been growing at a slower pace than what its emergency demands[1]. The lack of aggressive pursuit in development of new technologies is handicapping the lifesaving potential of the solutions we currently have.

Preservation conjugates numerous areas of transversally applicable knowledge: engineering, mathematics, thermodynamics, biophysics, biochemistry and material science are broad examples[6]. The disperse expertise required makes grant proposals very difficult, as institutions are logistically segmented to fund a specific discipline[6]. Ultimately, neglecting the preservation field not only masks its true importance but also deflects back to funding agencies, as they benefit less than they could if all other research fields did not have preservation difficulties.

Without institutional barriers, preservation science can thrive in all of its cross-disciplinary research: organ cryopreservation[1], the elucidation of organisms that can naturally enter biological stasis (also called "suspended animation") at subzero temperature environments[7] and new perfusion technologies[8]. All these sub-fields have been growing due to research breakthroughs in nanotechnology, genome sequencing, microscopic imaging, omics exploration and several others, and it becomes clear that retribution would also occur if preservation had direct funding.

The combined effort of scientists, physicians, advocacy groups, academic and industrial institutions, policymakers and government representatives aims to build a massive platform for preservation advances that enables the transport, repair, banking and function enhancement of invaluable tissues and organs, undoubtedly valuable in backstage research and front-end medicine.

1.2. Unmet needs

The United Network for Organ Sharing (UNOS) reports 20% of patients in its waiting list die before receiving an organ[9]. A suitable organ donor can provide at most eight viable organs, but statistics show only three of them are actually given proper use. Given only 0.3% of the US population that dies each year is suitable for organ donation[1], only 3,052 organs/year are available when there is a demand of at least 50,600 organs. Patient matching algorithms give priority to geographical parameters to avoid long transportation times, but immunologic mismatching is common. A

lifelong regime of immunosuppression can be adopted, but increases the risks of infection-related diseases and cancer[10] down the road.

The process of organ assignment is very chaotic as a consequence of time. Intensive research on organ perfusion[8,11,12], organ cryopreservation[13,14] and mechanistic studies of metabolic dysfunction[7,15] have served as pillars to optimizing organ preservation, but so far, maximal organ viability is still a matter of hours. The Nebraska Organ Recovery Foundation reports organ shelf-life under cold ischemia averages at 4 hours[16]. Using low temperatures allows to extend this time window to about 24 h, or 72 h with perfusion techniques. Lower temperatures alone are not sufficient to avoid ischemia-reperfusion injury (IRI). Also, upon transplantation, the organ must be able to withstand a blast of inflammatory cascades and oxidative stress that further contributes to early dysfunction and failure.

While the medical motto has become "the sooner the transplant, the better", it evidently shows our lack of control of important biological events. Besides the emergency of organ deficit, a lack of preservation knowledge has led to drawbacks in important fields that otherwise could be thriving[1]:

- Biobanking of organs, tissues and cells can impact cancer research and treatment by increasing sample availability. The ability to endlessly preserve valuable study models "buys time" in the race between knowledge acquisition and unwanted global aftermaths. This sort of time capsule enables the avoidance of catastrophic outcomes that would require full comprehension of its science before being acted upon.

- In drug discovery, research and development costs are severely inflationated due to the lack of readily available human tissue models. Results on animal models are descriptive but unreliable as some behaviors cannot be extrapolated to human patients. Simultaneously, studies on animal models are usually frowned upon by ethical concerns, further delaying potential breakthroughs.

- Excess availability of tissue and organs can sometimes be an emergent necessity. If preservation methodologies allow for a quick restitution of the much-needed biological samples, events like natural disasters or multiple car crashes involving several victims could ease the burden on local hospital resources.

This race against time is still an institutional chokehold leading to an inefficient system. By implementing successful strategies of long-term organ storage and viable transplantation-on-demand, we can facilitate the donor-acceptor matching process. Institutions could have time to organize patient listings and redefine selection parameters, in the hope the whole process becomes more generic, standardized and faster, but with increased compatibility at the same time.

1.3. A translational science: multidisciplinary efforts

Preservation is closely connected to other major fields of biomedicine, so it is considered to have a translational character. This means that any advances in preservation can simultaneously benefit all other fields that require storage of their resources. Attenuating this crisis to manageable proportions relies on a coordinated effect that involves tissue engineering, genetics, regenerative medicine, drug discovery, transplantation methodologies and preservation techniques. Contributions have been made to bioimaging[17], organ-on-a-chip devices[1], high-

throughput screenings[6], microfluidics[18], nanotechnology[19,20] and molecular genetic engineering[21,22].

Although sideline technology as once thought, preservation now proves to be a very central and pressing necessity in biomedicine. Currently, preservation aids in overcoming the following challenges:

- **Regenerative medicine:** the inability to long-term preserve artificial models severely affects productivity: production costs increase, quality control is meaningless, funding decreases and companies are unable to thrive[23]. Skin grafts can only be kept for a couple of days as preservation is the limiting factor[24].

- **Protection of reproductive cells and tissue:** patients undergoing chemotherapy or irradiation can suffer hormonal imbalance or lifelong infertility[14]. The ability to remove, preserve and re-implant reproductive tissue empowers medicine. More than 60 women have banked ovarian tissue before cancer treatment and still had healthy offspring after re-implantation[25].

- **Public health emergencies:** multiple-trauma accidents, natural disasters, radioactive explosions or biological warfare motivate the maintenance of large inventories of bone marrow, blood bags of all types, hematopoietic stem cells and every type of organ and tissue to avoid mass mortality[24,26,27]. There have been some advances with cryopreservation: shelf-life of different tissue samples was increased, and less incidence of complications post-transfusion were observed[28]. However, shelf-life is still not in the long-term range as idealized and post-thaw loss of viability is still an issue[29].

- **Acute injury transplantation:** about 30,000 limb amputations occur per year in the US and 66% of those pertain to children and young adults alone[30]. Efficient preservation techniques can increase reattachment success dramatically: hand[31], limb[32] and whole facial tissue[33] transplantation has already been possible for almost two decades.

1.4. Applications

Long-term interests involve R&D advances in xenotransplantation, artificial organ production based solely on organic materials and – the focus of this thesis – cryobiology. Perfusion-based techniques provide a flexible approach to several problems. Besides providing cold storage conditions that increase shelf-life and allow long-distance travel[34], biomarker flushing can be done to either increase organ performance or assess its conditions[35,36]. However, only cryopreservation can enable long-term storage in biological collections known as biobanks[37].

Biobanking organs at extremely cold temperatures allows much more than just efficient storage: tissue biopsies can be done to check for normal histology, metabolic function and associated conditions that might have been carried by the organ donor, like HIV[38], which is 10,000-fold more likely to be transmitted through an organ (1%) than by a blood transfusion. Ultimately, if organ shelf-life is extended from hours to only 6 weeks, the full battery of tests required to screen most diseases could be performed[39].

Table I.1. Coverage of transplantation needs by preservation strategies. Cryobanking yields the greatest impact on the resolution of organ deficit, but other forms of preservation are still essential in current clinical practice. *Adapted from Ward et al*[40].

Objective in transplantation needs	Perfusion	High subzero storage	Cryobanking
Expanding the donor pool	x		
Ex vivo functional assessment	x		
Improving transplant outcomes	x	x	x
Disease screening	x	x	x
Reduced post-op complications	x	x	x
Improved patient matching	x	x	x
Extinguishing immunorejection	x	x	x
Removal of geographic constraints	x	x	x
Flexible surgery scheduling		x	x
Backup transplant organs			x
Off-the-shelf organ availability			x
Fertility protection in cancer cases			x
Global organ matching			x
Xenotransplantation supply chain			x

Cryobiologists have long embraced the ideology of Alcor Cryonics®, which has already started cryosleep procedures with volunteer humans[41] in an attempt to achieve whole-body preservation. What started as a futuristic ideal of human race perpetuation is now led by a pressing matter in global health. We are on the brink of a new age for preservation science – with increasing scientific knowledge, improvement of technological sophistication and abolition of institutional and financial barriers, biomedicine will be fundamentally revolutionized.

1.5. Realms of research

Preservation science has branched into two main fields: methodology and formula optimization[42]. Methodology encompasses the creation of new preservation techniques and the exploration of different temperature ranges and types of automation (Figure I.2).

Cryogenic storage at -80ºC and below is currently done at biobanks to a limited extent, because preservation techniques still have not reached excellence. Thus, seeds, cells and small tissues are currently the only resources able to be efficiently stored. From a clinical perspective, organ perfusion still remains the easiest choice in battling this crisis: although it only provides temporary preservation, it does so with the best efficiency.

Organ perfusion was first conceptualized in 1812 by Cesar Julien Jean le Gallois in the form of extracorporeal circulation, known currently as *ex vivo* perfusion[43]. For more than 150 years, perfusion was done in normothermic conditions as professionals believed mimicking the physiological temperature was the best way to secure viability[44]. Experiments in the 1960s used cold serum as perfusate, and perfusion time could be increased from hours to days[45] without organ failure. Since then, researchers adopted consistently lower temperatures of preservation to mitigate metabolic-related damage (see Chapter I.3).

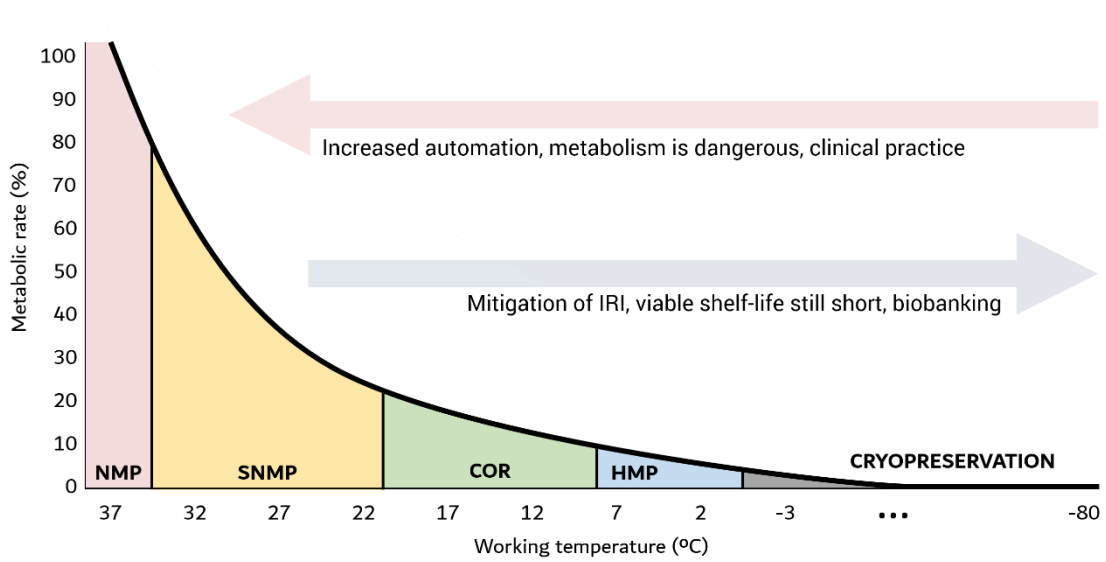


Figure I.2. Preservation techniques developed over the years span over a wide temperature range. Research usually goes back and forth between normothermic and subzero ranges to solve different problems. NMP = Normothermic machine perfusion (35–38ºC). SNMP = Subnormothermic machine perfusion (20–34ºC). COR = Controlled oxygenated rewarming (8–20ºC). HMP = Hypothermic machine perfusion (0–8ºC). *Adapted from Giwa et al*[1].

In the pursuit of biomedical excellence, several techniques have been designed to efficiently preserve organs and tissues alike. Currently, they are referred to as proofs-of-principle because all have had some successes but none has been adopted as a universal solution in the preservation challenge:

- ***In situ* organ preservation:** the donor body is used to keep the target organ alive while any sort of physicochemical stimuli are employed. For instance, inducing mild hypothermia in the deceased donor's body enables preservation of scavengeable kidneys while they still are in their natural physiological environment, which results in the increase of graft function right upon transplantation[46].

- ***Ex vivo* organ life support:** known simply as organ perfusion, *either* hypothermic[8], subnormothermic[47] and normothermic[48] automated perfusion devices can secure short-term viability of either thoracic or abdominal organs and eventually assess organ function prior to transplantation[49].

- **High subzero preservation:** temperatures lower than hypothermia are lethal if the organ is left unprotected. Like in perfusion, a cryoprotective fluid is used but the process is not dynamic, which means the organ is flushed but there is no continuous flow. So far, rabbit kidneys were preserved at -45ºC and remained functional upon transplantation[13] and supercooling of rat livers at -6ºC has extended shelf-life by at least 3-fold[47].

- **Programmed metabolic suppression (PMS):** to engage in induced hibernation, PMS relies on controlling chemical stimuli to capitalize on the biochemical and genetic resources the body has. Like the arctic wood frog, there seems to exist a regulatory gene for metabolic arrest conserved in the genome of humans[15,50]. Also, pharmacologically induced hibernation has been demonstrated in mice[51], which must indicate that dormant genetic information can be tapped in order to acquire cold resistance.

- **Cryopreservation:** the most common method for biobanking and experimental research. Cryopreservation has allowed conservation of human fertility[52], successful transplantation of amputated limbs[53] and organ storage at -140ºC with preserved function after thawing[54]. Realistically, successful preservation has been reported to range from weeks[53] to decades[34]. Current research focuses in cryopreserving samples in the full absence of ice, which could reshape the future of morphogically-sensitive tissue like blood vessels[55], corneal tissue[56] and cartilage[57].

The performance of any methodology mentioned above can only be maximized by implementing efficient cryoprotection fluids in the process (Chapter I.5). But before it is described what constitutes these solutions, it must be understood what physically and chemically nefarious phenomena they try to counteract.

2. Mechanistic features of ice growth

In preservation, the main concern is the formation of ice crystals with a decrease in temperature, as biological samples are mostly composed by water. To produce the smallest crystal permissible by entropic laws (5 nm), a minimum of 275 water molecules are required[58]. The great quantity of water present in living organisms essential for its survival is also its downfall, as physical injury can be immense.

The coldest temperature ever recorded in natural habitats was -94.7ºC in eastern Antarctica[59], but some organisms are still able to prosper in such an environment. This suggests that living creatures were selectively pressured to develop mechanisms of cryoinjury avoidance. The species *Cucujus clavipes puniceus* can be supercooled -70ºC without exothermic indicatives of freezing, even when cooled to -150ºC[60]. The stonefly *Nemoura arctica* from Alaska has been found to produce 3-fold more glycerol when left at -15ºC for almost 3 weeks[61]. The arctic wood frog excretes high amounts of sugars that envelop its body at -20ºC, leaving it in biological stasis from weeks to months[7].

These strategies are proof of remarkable evolutionary steps and preservation science tries to mimic these processes by exploring cryoprotective constituents capable of yielding similar survival rates to complex tissue systems.

2.1. Nucleation and growth

Ice formation is characterized by a nucleation and a growth event. In an aqueous solution, a decrease in temperature causes molecules to tightly pack into small agglomerates at a nanometric scale. Due to a higher vapor pressure and sharp surface curvature, they have a lower melting point than fully grown ice, so their presence is transient and of random occurrence. This is the reason why ice nucleation is said to be a stochastic event, because it relies on random ice nucleation cores appearing and dissolving while temperature decreases. In pure water, ice growth may not occur until *ca.* -40ºC is reached, known as the homogeneous temperature[62], and the solution will remain in a supercooled state during the whole process.

When a heterogeneous nucleator is present, like a salt or any molecule other than water, ice growth can still occur but at a higher temperature, as heterogeneous crystals have lower periodicity and therefore require less energy to break apart. When a given temperature is reached, the crystallization process begins. While ice nucleation relies on molecular motion, ice growth

depends on the diffusion of water molecules to the crystalline matrix and an outwards diffusion of solutes that are not to be incorporated in the crystal. Ice growth is then inversely proportional to the viscosity of the solution, which is why some cryoprotective agents are very viscous to hinder ice growth.

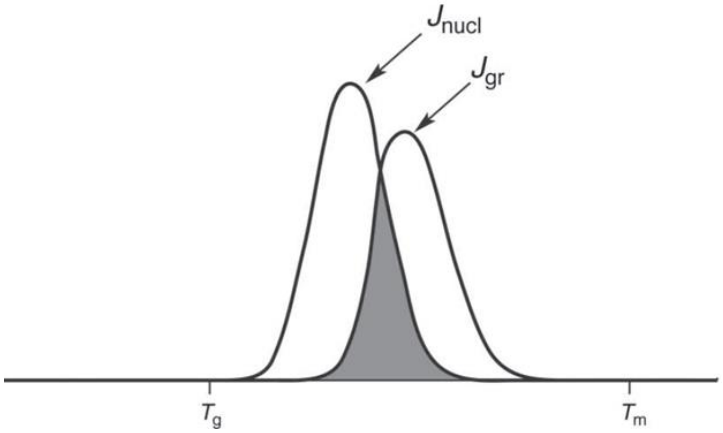


Figure I.3. Schematic representation of nucleation (J_{nucl}) and crystal growth (J_{gr}) rates at a given temperature range. Notice these events are dependent on the free energy of the system, thus the bell curves can be interpreted as probability of occurrence. *Adapted from Ramos et al*[63].

Figure I.3 represents the nucleation and growth rates at a given temperature, ranging from the melting temperature to the glass transition temperature of a system. Ice nucleation depends on the free energy (ΔG) between the ice and liquid phases in solution, which means that a chemical potential drives the process. A temperature decrease results in a higher nucleation rate (J_{nucl}) until it tends to zero near T_g, a glassy state with a reduced degree of motional freedom[64]. As the evolution from nucleation to growth requires the ability for molecules to reorient themselves, no growth nor nucleation can occur below T_g. Growth is more severely impacted by solution viscosity, so its bell curve is nearest to the T_m, where mobility is highest[65].

In summary, decreasing the temperature from T_m to T_g results in both nucleation and growth. Nucleation can still occur at lower temperatures as less molecules are involved in the process, but their lower melting temperatures means that no nucleation events are present near T_m. The opposite is observed for growth, occurring at higher temperatures but being hindered when viscosity increases. If the cooling rate applied to a system is high enough, it can bypass the growth stage and reach T_g with minimal amounts of ice nuclei, and this is the reasoning behind rapidly cooling biological samples by plunging into liquid N_2.

2.2. Critical radius for lethal crystals

Using fast cooling rates is mostly a technical cheat in the attempt to avoid ice growth, but some ice nuclei can still form and remain inside cells. When temperature increases again, the damaging process known as recrystallization can occur, and is detailed in Section I.2.3. However, the lethality of a crystal is determined by its size, and if a cooling rate is high enough, the ice nuclei formed may not have the time to grow to an extent that is damaging to a cell.

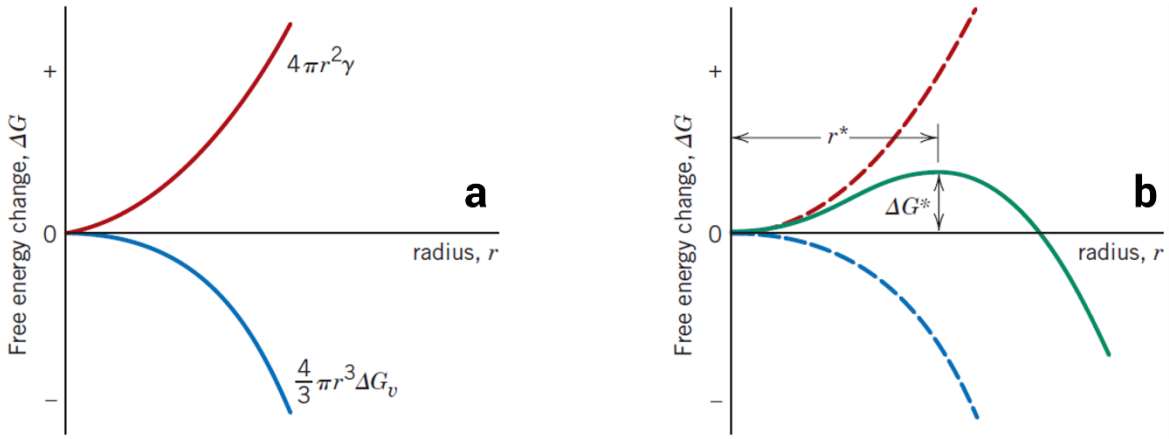


Figure I.4. Crystal growth is a trade-off between interfacial energy (red) and ΔG of the solid-liquid phase (blue). As a result of the dynamic behavior observed in (a), an activation energy (green) that separates nucleation and growth in (b) can be delimited by a critical energy requirement ΔG* and a critical radius r*. *Adapted from Callister et al*[66].

Figure I.4 shows that the threshold between nucleation and growth lies on the radius of the ice nuclei in solution. The development of an ice nucleus is a trade-off between its surface energy and the ΔG of the system that results in an activation energy barrier. Given enough size, the energy barrier that prevents growth from occurring is surpassed and this point is referred to as the critical radius (r*).

Nuclei radius is a determining factor to the survival of organisms, either in crystallization upon freezing or recrystallization upon thawing. The formation of ice nuclei is not fully avoidable, but organisms can still survive when crystals are about 100–300 nm in size. At a dimension of 400 nm, crystals become so damaging to the cellular structure that eventually lead to death[67].

2.3. Physical cryoinjury

Simply freezing a sample is not error proof, and molecules known as cryoprotective agents (CPA) must be present to ensure ice damage is minimized (Chapter I.4). Lowering the temperature causes a wave front to form as the crystal lattice grows and engulfs the liquid medium (Figure I.5). This causes solute concentration gradients and can mechanically damage large structures by applying shear stress. The intensity of the wave front depends on the cooling rate applied:

- If cooling is too slow, cells exposed to the CPA will shrink as water leaves the cell to hydrate the newly added solute, decreasing the water fraction that can freeze intracellularly. Thus, extracellular ice formation (EIF) occurs and crystals of damaging size mechanically disrupt the cell membrane from the outside. The wave front will force residual water and the CPA to co-diffuse to the cell. Until the water-to-ice transition halts, CPA will become more concentrated over time until viscosity is so high that molecular mobility is impeded, and crystals cannot form anymore: this is called solute hypersaturation and is the basis for vitrification[68].

- If cooling is too fast, the CPA does not have time to enter the cell before low temperatures stop any diffusional action, so the hypotonic state of the cell drives intracellular ice formation (IIF) to occur. Any intracellular nuclei that did not evolve to fully grown crystals are able to further damage the cell when thawing occurs.

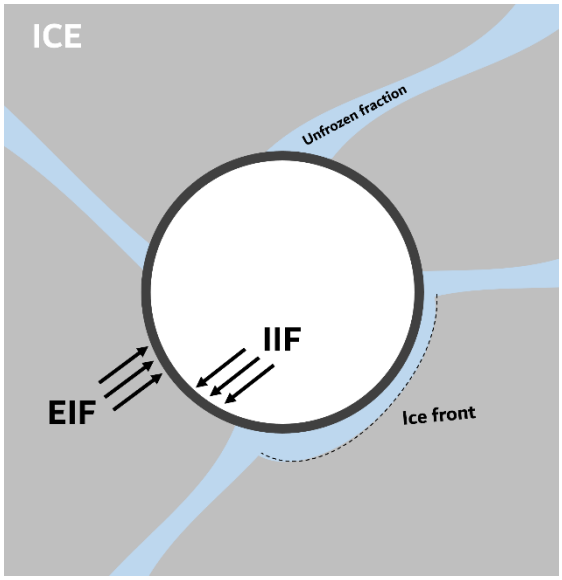


Figure I.5. Single-cell representation of the possible physical stimuli freezing can generate. Ice forms around cells by gradually advancing as a wave front, leaving only residual fractions of unfrozen liquid mainly composed of hypersaturated solutes so viscous that ice formation is impeded. Depending on the cooling rate applied, the cell membrane can suffer physical stress from either intracellular or extracellular ice formation. *Adapted from Hubel*[69].

Upon thawing, further damage can occur. For instance, a slow cooling rate that resulted in solute hypersaturation will cascade into posthypertonic osmotic shock: the ability of extracellular water to diffuse abruptly into the cell will create a gradient too aggressive for the cell membrane to withstand, resulting in cell lysis. Likewise, fast cooling rates could not prevent ice nucleation that, given enough water mobility, can recruit water molecules for crystals to grow once more, this time to lethal dimensions. This process is called recrystallization and is colloquially defined as the silent assassin of freezing procedures[4].

3. The Hypothermic Continuum

Freezing a biological sample is by far considered the best preservation method. For a 10ºC decrease, cell metabolism drops by 50%, which means one single ion would take over 1 hour to diffuse through the membrane at -80ºC. However, freezing only provides passive protection, as the cell must also deal with chemically generated attacks when thawed due to loss of homeostasis.

The hypothermic continuum (HC) is a chain of events that describes how a biological system reacts at each step of decreasing the temperature from 37ºC to near subzero temperatures (0–10ºC). With a gradual decrease in temperature, the loss of kinetic energy necessary for chemical reactions results in biochemical pathway decoupling, which leads to the depletion of adenylates due to the failure of aerobic ATP production. Membrane-mediated transport becomes disrupted and all ionic gradients destabilized, which leads to intracellular increase of Ca^{2+} and Na^{+} while K^{+} decreases, ultimately leading to intracellular acidosis (pH *ca.* 4)[70].

Simultaneously, cell and organelle membranes undergo lipid phase-changes from the liquid-crystalline state to the solid gel state. A 10ºC decrease results in 3% of thermal energy being dissipated without contributing to metabolism, so membrane-bound enzymes that adopt high-energy conformations to function are not supplied with the required activation energy. Fluid leakage starts to occur, further contributing to diffusional imbalances[71]. All these disruptive events will compromise the integrity of the cytoskeletal matrix necessary for volume change adaptation[70].

3.1. Mitochondrial Permeability Transition Pore Opening

A state of hypothermia has delayed consequences on re-warming. Even if cryoprotective agents are used to avoid lethal ice growth, the overload of oxidative stressors that originate from this hypothermic disruption provide multiple routes for gene-regulated cell death to occur as soon as the cell is thawed. The diffusional impairment created by the hypothermic continuum results in what is called the mitochondrial permeability transition (MPT) pore opening event.

Because transmembrane ionic gradients are lost due to lack of mobility, the membrane redox potential that acts as a barrier to passive diffusion is not present when the cell is thawed. Thus, compartmentalized molecules can wander freely throughout the cytosol, and when metabolism kickstarts again, a cascade of unwanted reactions starts to occur.

The mitochondrial pair Bcl2/Bax ratio of anti- and pro-apoptotic proteins decreases and cytochrome C is released into the cytosol to activate the apoptotic cascade[72]. A high flow of reactive-oxygen species (ROS) populates the cytosol and activates caspases-8/9 responsible for apoptosis[73,74].

3.2. Ischemia-Reperfusion Injury

In a multicellular context (tissues and organs), the combination of both physical and chemical injury is known as ischemia-reperfusion injury (IRI). In this case, the cardiovascular system also plays a role in how cells react to hypothermia.

IRI is usually studied under perfusion conditions, in which an organ is flushed with a cryoprotective solution (Section I.5.1) while undergoing hypothermia and washed when temperature is returned to physiological conditions. Thus, the organ undergoes a bimodal state: one of ischemia, when blood supply is deficient; and one of reperfusion, when blood flow is re-implemented to resuscitate the organ[75]. Figure I.6 summarizes the full collection of stimuli cells undergo during preservation.

The ischemic insult largely depends on time, which means that tissue damage is a cumulative consequence of the absence of blood flow. During ischemia, cells rely on anaerobic metabolism to survive, as O_2 is equally deficient in the system, which leads to respiratory acidosis. To counteract this event, the Na^+/H^+ exchanger starts influxing a large amount of Na^+ that cannot be balanced due to ATP depletion, leading to inactivation of Na^+/K^+-dependent ATPases. An increase of intracellular Ca^{2+} occurs, leading to the aforementioned MPT pore opening event. These events will induce different changes on phenotypically variable cells: for example, the heart will suffer intracellular calpain activation, a protease that degrades myofibrils and results in hypercontracture, much like what occurs in endothelial vessels[76].

When the organ is reperfused with blood, O_2 and nutrients are efficiently delivered, and aerobic respiration can initiate. However, there is a major outburst of accumulated H^+ to the blood stream to restore physiological pH which can lead to major imbalances. For instance, acute intake of O_2 can result in ROS generation which, when paired with Ca^{2+} accumulation and MPT pore opening, can lead to apoptotic or necrotic cell death. Inflammatory cascades, pathogenetic responses and sudden excretion of metabolic waste are also contributing factors to decreased survival.

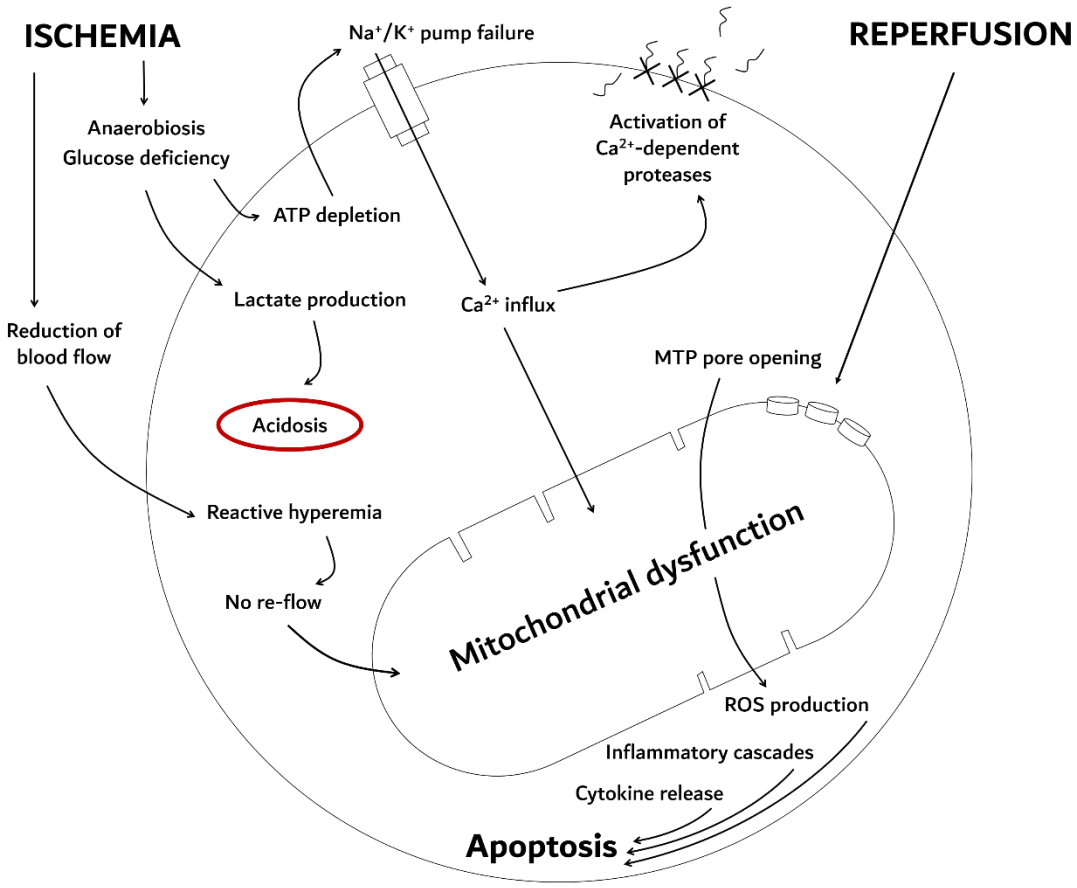


Figure I.6. General overview of the events associated with ischemia-reperfusion injury. Some parallelisms to the HC and MPT pore opening can be drawn, as they all result in physical and chemical damage regardless of sample type. Notice that cryoinjury is not represented but is occurring simultaneously.

Paradoxically, reperfusing an organ with autologous blood can exacerbate tissue injury beyond osmotic damage if the carrier solution previously used does not precondition the organ to sustain such flow. Cell death can continue for up to 3 days after the onset of reperfusion.

In summary, biological dysfunction is an unavoidable consequence of cryopreservation techniques, but it can be alleviated by using cryoprotective agents and solutions.

4. Strategies for mitigating cryoinjury

The presence of different substances in water can induce changes in melting and freezing temperatures, which is closely related to changes in crystal structure. CPAs are substances capable of interacting with ice nuclei and hinder the recruitment of subsequent water molecules. They can also provide a viscous system in which crystallization is harder to occur. There are also substances that can act as ice nucleators by enabling crystallization to occur faster, resulting in crystals with reduced dimensions or harmless shapes.

4.1. Chronological discovery of CPAs

A cryoprotective agent is a substance capable of affecting crystal formation. In a cellular context, it can be penetrant like DMSO and glycerol or non-penetrant like most polymeric structures. Likewise, the latter can be of synthetic origin, such as polyethylene glycol (PEG, 0.2–9.5 kDa),

polyvinyl alcohol (PVA, 30–70 kDa) or hydroxyethyl starch (HES, 130–200 kDa); or naturally produced, such as mixtures of alginates and chitosan, various sugars and proteins.

Glycerol was the first molecule discovered to have a cryoprotective effect back in 1949 by Polge *et al.*[77], when he successfully cryopreserved spermatozoa cells during long time periods. Due to its viscosity, it hinders ice formation, and because it is an electrically neutral molecule, it balances the overall electrolyte concentration in the residual unfrozen medium after freezing has occurred.

Glycerol usage kickstarted research on cryobiology and its effectiveness was only surpassed ten years later with the discovery of DMSO[73]. DMSO was synthesized by Zaytsey in 1866 but its use as a cryoprotectant was only explored almost a century later. Because it is a low-cost reagent and presents the lowest cytotoxicity among all CPAs known, it has become the gold standard in biological storage solutions[78].

4.1.1. *Ice blockers*

In the biological milieu, the huge diversity of molecules can greatly increase the probability of heterogeneous nucleation, resulting in large crystal formation. Therefore, cryoprotective mixtures are usually supplemented with ice blocking additives. 21st Century Medicine is a R&D company that specializes in synthesizing CPAs and additives that ensure that least amount of crystal growth.

Polyvinyl alcohol (codename X-1000) and polyglycerol (Z-1000) are sold as supplements to CPA usage[79]. Organic chemistry plays an important role in synthesizing new CPAs with tailored properties. Lately, Ben *et al.* has contributed remarkably[80] by producing photoactivatable switches that are able to block or not interfere with crystal growth at will.

4.1.2. *Polymers*

Most polymers are non-penetrant, so their function is not related with diffusion. Instead, they regulate osmotic flow and balance out the osmolarity of internal solutes. Polymers are an attractive study object, as their variable size and composition can influence how they behave in aqueous media, thus yielding variable effects.

Antifreeze proteins (AFP) are the most remarkable product of natural adaptation to cold environments. Organisms that inhabit subzero temperature environments have developed acclimation mechanisms based on expressing proteins that circulate in blood. Celik *et al.* demonstrated that AFP binding to ice hinders growth while blocking Ca^{2+} and K^{+} ion channels, maintaining membrane stability.

In general, antifreeze proteins have two advantages: they can provide a similar protection with a concentration 300–500 times lower than a CPA; and do not present cytotoxicity issues, as they are controlled by genetic regulatory action[81]. However, their purification or *de novo* synthesis for implementation in cryopreservation becomes an expensive endeavor and other solutions are usually explored.

4.2. Disadvantages of CPA usage

Cryoinjury comprises a big number of consequences on cell morphology and function, and employing cryoprotective substances can minimize those effects, but the cooling and warming rates applied also play an important role on how these constituents interact with the cells.

4.2.1. *Osmotic solution effect*

Spermatozoa cells are remarkable study models in cryobiology as they can withstand greater volume changes than any other cell, as they are rich in fatty acids that maintain osmotic pressure, hence their high viability even when membrane stiffness is partially lost after the thawing step[82].

However, most cells require that the freeze, thaw and washing of the CPA processes encompass slow diffusional rates: although an animal cell can shrink to about 5-fold its original size, it can only inflate 1.5-fold before lysis occurs[82]. The decrease in temperature further decreases membrane flexibility, which hinders cells from correctly adjusting to sudden volume changes.

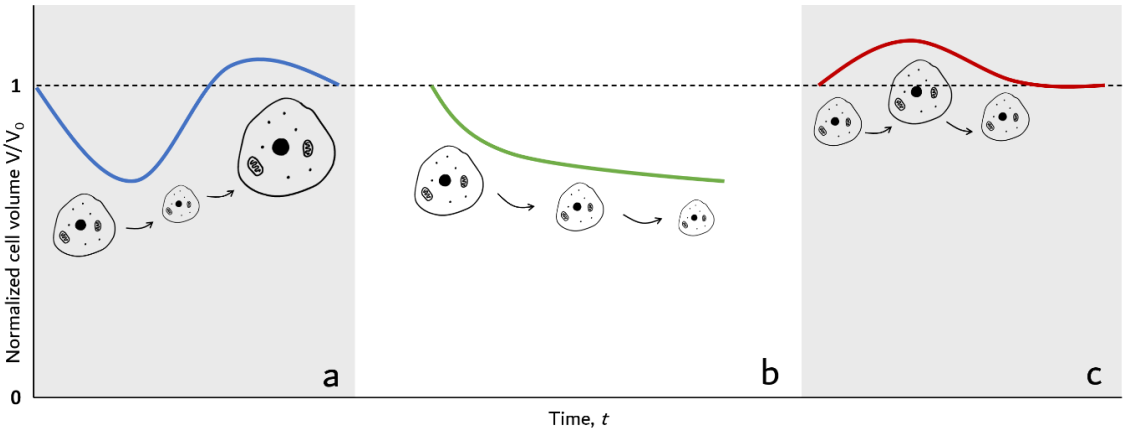


Figure I.7. Volumetric cellular response to different stimuli. In (a), a penetrating CPA is added. In (b), a non-penetrating CPA is added. In (c), the supplemented CPA is washed upon thawing. Cell shrinkage is represented as a reduction of spherical volume, but realistically, the cell membrane collapses inwards due to cytoskeleton action. *Adapted from Hubel*[69].

As a result, the sole use of CPA is not enough to guarantee regular cell volume during freeze-thaw and the addition of impermeant molecules able to act as osmotic regulators and balance the effect of CPA injection and removal is required. A mechanistic example of a sectional slice of a blood vessel surrounded by tissue is shown in Figure I.8.

Initially, the system establishes a diffusional gradient of water and ions between the blood stream and tissue cells to maintain an isotonic state. Upon injection of a CPA solution, water will enter the blood stream to counteract blood vessel hypertonicity. If the CPA is dissolved in an isotonic buffer solution, cell volume will be maintained, and osmotic effects can be avoided. However, a hypotonic buffer will result in osmosis to tissue cells, which can lead to cell bursting.

In a low-temperature environment such as hypothermic perfusion, diffusion is greatly hindered, which potentiates solution-based cryoinjury. In cryogenic conditions, the greater amount of water in the tissue will provide significantly big ice crystals that will rupture the cells beyond the cryoprotective ability of the CPA. Freezing of the aqueous medium that results in solute hypersaturation further leads to more aggressive osmotic gradients.

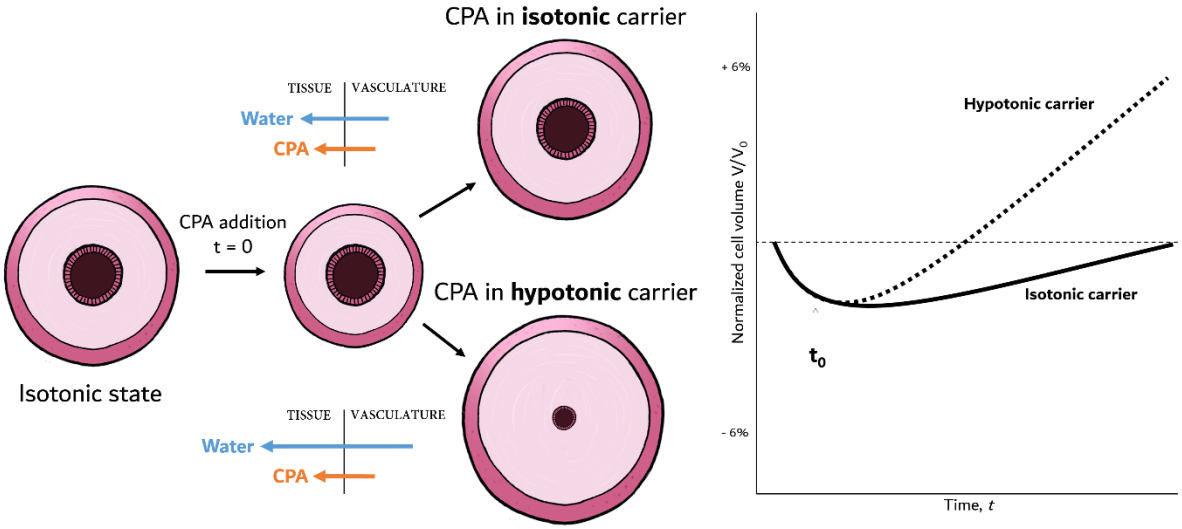


Figure I.8. The volumetric response in tissues is similar to cells but involved equilibrium with the vasculature. Without proper tonicity balance, tissue edema can severely compromise viability. The inner circle represents a blood vessel surrounded by proximal tissue.

4.2.2. *Cytotoxicity*

It is common to believe that an isotonic buffer is unnecessary if CPA concentration is simply increased: viscosity increases, molecular reorientation is harder, crystal formation is avoided, and cell shrinkage is a tolerated collateral effect (as ultimately, less water is freezable). In fact, high concentrations of CPA must be used for the desired effects, which means CPA diffusion to the intracellular space is greater, but this results in CPA-induced cytotoxicity. In 1966, Meryman *et al.* first discovered the adverse effects of DMSO on cell viability. Fahy *et al.* confirmed that DMSO resulted in poor heart recovery and observed that when both frozen and unfrozen tissue slices were exposed to DMSO, the extensive ice formation alone could not fully explain the viability reduction obtained[83].

Essentially, high CPA contents can bypass the cell constraints associated with temperature drops and adverse osmotic effects, but they can become toxic (about 1.9M for DMSO[83]) to the extent of enhancing cryoinjury instead of avoiding it. However, toxicity is not simply due to high concentrations but to an osmotic change. At physiological temperature, high concentrations are not sufficient to be toxic, but at -5°C, the cell is less tolerable to volume changes, and the osmotic effect is enhanced to a point at which it is toxic[83]. In fact, at -50°C, it becomes hard to discern between ice-associated and CPA-associated cryoinjury: in this range, diffusion is hampered and toxicity-borne effects are not as fast.

As stated by Greg Fahy[84], *"Whereas pharmacologists have drug antagonists, and biochemists have enzyme inhibitors, cryobiologists have lacked antidotes to cryoprotectant toxicity for the most part"*. Currently, there is no molecule nor mechanism capable of neutralizing the intrinsic cytotoxicity of DMSO and all other CPAs used[83]. However, some strategies have been implemented in the search of cryoprotectant toxicity neutralization (CTN).

CPA co-dilution is based on using a mixture of two different CPAs, halving their effective concentrations in solution (and their toxicity) but maintaining an adequate cryoprotective concentration. Leibo and Mazur reported an increase in viability when DMSO+PVP mixtures were used[85], but the results were questionable: rather than an intrinsic CTN effect, viability increase

could relate to (i) mutual dilution to sub-toxic concentrations during freezing[84], (ii) altered phase-diagrams or (iii) a sum of beneficial cryoprotection mechanisms.

There is a strong correlation between toxicity and the strength of hydrogen bonding between water and CPAs[86], as shown in several amide reactivity studies[84]. For DMSO specifically, reports postulated it could permeabilize the cell membrane to allow diffusion of impermeable substances, thereby disrupting the transmembrane gradient and ultimately leading to cell death[87]. Also, DMSO causes sulfhydryl oxidation of proteins, which results in proteinase overexpression[84].

When rabbit renal cortical slices were incubated with VS41A (containing DMSO), Na^+/K^+-ATPase activity (an SH-dependent membrane protein) reduced by 30%, which could be reversed by adding DTT[88]. However, the Na^+/K^+ gradient could not be fully replenished, indicating that other effects are occurring. Sulfhydryl reagents are known to be mitochondrial poisons, so it is possible that mitochondrial membrane damage (which suffers permeability depression during the freezing process) might have occurred.

4.3. Carrier solutions

Given these disadvantages, the sole use of a CPA in a biological system is somewhat insufficient. Cells must be stabilized during the whole procedure and their nutritive requirements must be met. Therefore, cryobiologists have invested in developing different media compositions known as carrier solutions. As the name states, carrier solutions 'carry' a CPA as the active agent in a formulation, and the remaining constituents provide a functional backup to the several challenges a cell must face.

Depending on their use, these solutions can be normothermic, hypothermic or cryogenic formulas and their compositions are highly variable, although some common criteria are met (see Section I.5.3). Of an immense diversity of compositions in the market, Euro-Collins[89], UW Solution[89], Custodiol-HTK[89], Celsior[89], ET-Kyoto[90], IGL-1[89] and Hypothermosol[91] are some of the most popular.

Table I.2. Summary of the composition of popular preservation solutions currently marketed. Values represent concentrations in mM.

Composition	Euro-Collins	UW	Custodiol-HTK	Celsior	ET-Kyoto	IGL-1
Na^+	10	25	16	100	100	125
K^+	115	125	10	15	44	30
Ca^{2+}	---	---	0.015	0.25	---	0.03
Mg^{2+}	---	5	4	13	---	5
Cl^-	15	20	32	71	---	---
Glucose	180	---	---	---	---	---
Colloid/Impermeant	---	Pentafraction, Lactobionate, Raffinose	Mannitol	Lactobionate, Mannitol	Pentafraction, Trehalose	Lactobionate, Raffinose, PEG
Buffer	Phosphate, Bicarbonate	Phosphate	HEPES	HEPES	Phosphate	Phosphate
Antioxidant	---	Glutathione, Allopurinol	α-ketoglutarate	Glutathione	---	Glutathione, Allopurinol
Amino acids	---	---	Histidine Tryptophan	Histidine Glutamic acid	---	---
Others	---	Adenosine	Deferoxamine, LK 614	Sucrose	Sulfate, Dibutyryl cAMP, Gluconate	Nitroglyceryl, Adenosine

Whilst some contain blood components, others can be completely serum-free and their use on cell/tissue or immersion/perfusion systems can vary.

5. Preservation formula development

Any technique mentioned in Section I.1.4 does not guarantee viability without a cryoprotective formula. While fast freezing macromolecules such as DNA could secure intact structure and function, matter at the cellular or organic level will not withstand cryoinjury when unprotected. Therefore, implementing a technique always brings about a cryoprotective formula tailored for that application and cell type.

5.1. From blood to blood substitutes

Before cell culture media were formulated in the 1950s, perfusion of *ex vivo* organs was done with blood-based perfusates. However, due to xenoimmunological and ethical concerns, these fluids had to be replaced by ion-rich solutions[92]. Using fetal bovine and calf sera in culture media was attractive due to the absence of antibodies that could instigate immune responses, but the ethical concerns overpowered any potential advantage, inciting the development of serum-free solutions.

Blood perfusates are pretty efficient in securing organ viability in normothermic perfusion: they have all the required nutrients and O_2 concentration to meet the metabolic demands of the organ. However, normothermic perfusion has some disadvantages that are carried over to hypothermic perfusion. For instance, blood is a non-Newtonian fluid that thickens exponentially when blood flow and temperature are reduced. As a consequence, the vessels will experience extreme mechanical stress and nutrient diffusion will be hindered. Other disadvantages of using blood perfusates in hypothermia include[93]:

- Nefarious immune-mediated responses;
- Hemolysis;
- Thrombus formation due to blood thickening;
- Biochemical/humoral variations leading to metabolic stress and genetic change;
- Increased risk for blood-borne infections.

All these factors motivated the development of acellular perfusates designed to ease the effects of the hypothermic continuum and avoid disadvantageous consequences that led to irreparable outcomes.

5.2. ICF and ECF-type solutions

After the Collins solution was made commercially available in 1969 for use in kidneys, hearts, livers and lungs, the addition of impermeant molecules to regulate osmotic shock improved overall chemical stability, and this new formula was sold as Euro-Collins[94]. For the first time, the organ became tolerant to cold ischemia for a prolonged period of time. Although each solution was initially formulated with the goal of preserving a specific organ, all of them have shown outstanding performance in any type of histology[95].

Despite the immense diversity of compositions around (Table I.2), they can all be classified according to their ability to mimic the extracellular or intracellular environment:

- **Intracellular fluid-type solutions (ICF)** are composed of low Na^+ and high K^+ concentrations, mimicking the natural ionic balance of the cell. These ions are mediated by active transport mechanisms, like the Na^+/K^+-ATPase, which has a lower turn-over rate at low temperatures[96]. This type of composition was intended for preventing cellular edema: upon dysfunction of the Na^+/K^+ pumps, injection of a hypotonic solution would result in the migration of water to the cells, effectively bursting it in the process of trying to balance ion concentration (as seen in Subsection I.4.2.1).

- **Extracellular fluid-type solutions (ECF)** originated from observing that high K^+ concentrations could increase the risk of hyperkalemia-induced pulmonary vasoconstriction[97]. ECF-type solutions have inverted concentration ratios (high Na^+ and low K^+) and are compensated by the addition of other ions, amino acids and histidine-based buffers.

Some formulas have balanced Na^+/K^+ ratios that are otherwise compensated by the addition of specific molecules to play a specific purpose. Usually, there is no limitation to the amount or type of substances that can be implemented, as long as specific criteria are fulfilled.

5.3. How to build a cryoprotective fluid

Formula optimization is mostly a trial and error process with the goal of achieving highest viability. As an example, the design rationale for developing the Unisol solution is hereby described, although the process is very similar for any other formula. Usually, a solution is initially envisioned for one of two applications: a flushing solution for static cold storage (SCS) or a perfusate for dynamic cold storage (perfusion). The Unisol solution is a prime example of hybrid functionality and therefore provides a universal baseline[70].

A cryoprotective formula aims to provide five basic functions: minimizing cellular edema, regulating osmolarity, counteracting acidosis, scavenging free radicals and providing substrates for a quick regain of high-energy products that stimulate cell recovery after thawing. Extra functionality is considered secondary and relates to any supplementary additions. The aim of Unisol specifically was to use multifunctional components to maximize viability while minimizing constituent amount, and therefore, cost of production.

Impermeant molecules for **osmotic regulation** must be present for the organ to withstand volume changes due to hypothermia. Thus, lactobionate (an impermeant anion) is used to replace the extracellular Cl^-. Reducing the extracellular amount of chloride will directly influence the balance of all other ions, with subsequent regulation of the oncotic pressure. Viaspan, Hypothermosol, Celsior and Cardiosol are examples of formulas that heavily rely on lactobionate for osmotic regulation[98]. Lactobionate can also chelate Ca^{2+} and Fe^{3+}, therefore contributing to reducing the calcium influx (that leads to apoptosis) and oxidative damage, respectively[70]. Gluconate is an equally effective alternative and is used in KPS1, marketed by Organ Recovery Systems, and mixtures of both are commonly used. To complement the osmoticum of Unisol (the collection of substances that contribute to osmotic regulation), sucrose and mannitol were used. Mannitol also scavenges OH^- radicals and induces prostaglandin-mediated vasodilation that reduces vascular resistance at low temperatures[99].

To maintain an oncotic pressure equivalent to blood plasma, polymeric oncotic regulators are used and referred to as colloid agents. Due to their size, they are unable to fenestrate through the capillary wall and leave vascular circulation. Thus, they maintain oncotic pressure at physiological range and are readily eluted when reperfusion is performed. Starch-derived polysaccharides,

gelatin polypeptides or nonionic detergent copolymers are candidates for colloidal function. In Unisol, dextran-40 was used, an α-1,6 glucose polymer of 40,000 Da capable of balancing hydrostatic pressure and preventing interstitial edema[100]. Initial research on dextrans demonstrated their ability to prevent erythrocyte clumping (reducing vascular resistance) and increase intravascular osmotic pressure, which is why they are widely used in formulas that require *in vivo* or *ex vivo* washout. Besides its function, dextran is used as a plasma expander that can be excreted by the kidneys[101] and can replace HES in the UW solution for kidney preservation[102]. Dextran has an advantage over starch-based colloids because the viscosity of the solution does not increase as much, providing laminar flow and preventing mechanical damage to the endothelial wall.

In regards to the **ionic composition**, the Na^+/K^+ and Ca^{2+}/Mg^{2+} ratios are of utmost importance. For instance, Unisol uses similar Na^+ and K^+ concentrations to restrict passive diffusional exchange when the ionic pumps are inactivated due to hypothermia. Viaspan and Euro-Collins rely on very high potassium levels that could induce hyperkalemia[103], so Unisol uses a lower concentration, but one that is sufficiently high to classify the formula as an ICF-type solution. Ca^{2+} was severely decreased in respect to Mg^{2+} as there is good evidence in myocardial preservation that survival increases in these conditions. However, Ca^{2+} must not be zero due to the putative calcium paradox, which states that a total absence of Ca^{2+} will result in injury similar to IRI, leading to myocardial lysis and heart failure[104].

Glucose is included as an **energy substrate**, but it must be kept at low concentrations to prevent cellular overload during hypothermia, as glycolysis can be shifted to lactate production in anaerobic conditions, leading to intracellular acidosis[70]. **As a countermeasure for acidosis**, a pH buffer that is effective in nonphysiological conditions must be included. HEPES is used given its optimal biocompatibility out of all aminosulphonic acid buffers that are optimized for low temperatures[105]. Because HEPES is a synthetic zwitterion of 238 Da, it also contributes to osmotic regulation from the extracellular space.

To support recovery upon thawing, adenosine is usually added to serve two purposes: (i) it is a substrate for ATP regeneration and (ii) enhances vasodilation to facilitate solution washout[106]. **To avoid oxidative damage**, glutathione is included as a cellular antioxidant that scavenges $OH^{\cdot}$ radicals. It is also a cofactor of glutathione peroxidase[107], protecting against lipid peroxidation by H_2O_2.

This collection of substances composes the backbone of preservation solutions to which any mixture of pharmacologically active substances can be added, depending on the type of tissue or preservation technique. Table I.3 summarizes alternative substances and additional categories that are relevant to optimize the preservation procedure.

Table I.3. Alternative reagents in formula development to guarantee specific functions[70]. The rightmost column shows additional functions that are worth considering when tailoring a formula for a specific role.

Impermeant	Colloid	Other supplementary functions
Citrate	HSA	Membrane stabilizers
Glycerophosphate	HES	High-energy substrates, such as nucleotide precursors
Gluconate	Haemaccel	Inhibitors of apoptosis
HEPES*	BASF	Vasoactive agents
	PEG	Hemoglobin-like molecules, capable of O_2 delivery
		Supplementary antioxidants
		Ca^{2+}-channel blockers
		Growth factors

*An example of an anionic form of aminosulphonic acids.

6. The field of cryobiology

Cryobiology is the field that studies life at subzero temperatures either at mild, hypothermic or cryogenic conditions. The field of cryobiology is very diverse, and is branched according to five different categories[108]: the object of study (animals, bacteria, macromolecules), the size and complexity of the sample (cells, tissues, organisms), the area of scientific interest (medicine, botanics, storage facilities), the sample type (stem cells, blood, solid organic matter) and the methodology (vitrification, anhydrobiosis, cold storage and others).

Recently, cryosurgery has been on the spotlight due to the ability of selectively destroying tumors in the near proximity of healthy cells. The *status quo* of cryobiology is not in a primitive state, but further knowledge is required and the sophistication of the current technology is its only limitation.

6.1. Research on cells

Most research in cryobiology done on cells has the sole objective of organ scale-up. However, some successes have been reported in specific cell type preservation. Stem cells comprise most of cell research due to their ability to greatly improve regenerative medicine. Goltsev *et al.* reported the ability of stem cells to differentiate into insulin-producing cells in both the pancreas and the brain became diminished if membrane damage occurred during freeze-thaw cycles[108].

On the same note, pancreatic islet transplantation may provide a cure for type I diabetes, but the survival rate of such cells when cryopreserved is still less than 50%[109]. Leukocyte banking has also improved. Svedentsov *et al.* implemented exponential freezing procedures in combination with less toxic CPA solutions, improving human leukocyte survival after both storage and retrieval[108].

Reproductive cells are also a big research interest. The first embryo was cryopreserved in 1996 in an attempt to enable *in vitro* fertilization of a patient diagnosed with breast cancer[110]. So far, cryopreservation has shown to enable normal function of sperm cells either as cell suspensions or fully grown gonads for patients that undergo cancer therapy or undesired vasectomy[108].

6.2. Research on tissues and organs

Organ preservation is currently the biggest challenge. Kidneys are the go-to model for cryopreservation experiments, as they tolerate ischemia better than any other tissue[4]. The first attempt dates back to 1973, when Dietzman *et al.* discovered that canine kidney perfusion with 12.5% DMSO dissolved in hemoglobin-free plasma while cooling to -22ºC could control the growth of ice crystals and ensure brief physiological functions upon thawing[78]. Six years later, Macklis *et al.* observed that using 10-15% DMSO to freeze a kidney to -79ºC yielded a 50% survival rate upon transplantation, blood flow could be maintained for 1 month and morphology was intact[111].

In 1997, Gregory Fahy, known as the pioneer of the vitrification procedure, proved that **kidney** vasculature could survive cryopreservation when the organ was perfused with a vitrifying solution and then frozen. Blood flow resumed immediately after rewarming and function was intact. After 3 weeks of auto-transplantation, still no renal damage was observed[112]. Fahy *et al.* continued his work and reported in 2004 that an optimized concentration of 8.4 M and further 9.3 M did not devitrify after thawing from -150ºC at a 1ºC/min warming rate, and the solution could be eluted in its viscous state whilst enabling full kidney recovery[13]. This discovery became the hallmark of cryobiology, despite being successful only once, as the results still have not been reproduced to

date. Nevertheless, it officially showed that successful organ cryopreservation and transplantation can be achieved given a very particular experimental setting.

Heart cryopreservation began earlier than kidneys. Connaughton and Lewis *et al.* inaugurated heart cryopreservation in 1961 by freezing two rabbit hearts to -21ºC with 10% glycerol, of which only one resumed cardiac pumping[113]. Robertson *et al.*[4] followed with 15% DMSO and stored the hearts for 2 h. Karow *et al.*[114] proposed in 1965 an ideal cryoprotective solution composed of 15% DMSO, 6% dextran and 110 mM Mg^{2+}. The solution had a strong cardio-protective effect on rabbit atrial slices frozen to -20ºC as survival rate averaged 80-93%. Co-diluting DMSO reduced cytotoxicity but organ viability was still reduced by impairing contractility and blocking atrioventricular blood flow.

As of 2016, whole **liver** cryopreservation is still unsuccessful, most likely because high metabolic work might severely affect post-thaw viability due to chemical imbalance. Moss *et al.* attempted glycerol cryopreservation in 1966 at -60ºC for two weeks, but after pelvic transplantation, all animals died within 6 h due to liver dysfunction[115]. Almost 30 years later, Wishnies *et al.* vitrified human liver slices with 4.76 M of 1,2-propanediol and enzymatic activity was preserved[116]. In the same conditions, Ekins *et al.* observed vitrified liver slices could still metabolize xenobiotics, but at a lower rate than its fresh counterparts[117].

Ishihara *et al.* demonstrated that cooling pancreatic islet cells at 0.5–1ºC/min and quick thawing successfully preserved islet cell activity[118], while Sandler *et al.* reported that a 5–25ºC/min cooling outperformed the previous results and did not result in any structural injury[119]. Warnock *et al.* undertook pancreatic transplants in diabetic rats and noticed that cooling at 0.25ºC/min and thawing at 200ºC/min after -196ºC storage for 2 weeks reversed the symptoms of pancreatic injury[4]. Hullett *et al.* followed shortly after by cryopreserving human fetal pancreas as usual and noticed full viability upon transplantation onto diabetic rats[120]. It was in 1989 when Kneteman *et al.* first proposed the use of DMSO in pancreatic research[121] and Yokogawa *et al.* demonstrated 10% DMSO conserves histology and insulin secretion activity of islet cells[122].

Sugimoto *et al.* discovered just 18 years ago that the endocrine function of cryopreserved infantile rat **ovaries** was identical to fresh ovaries[123]. *In vivo* maturation studies by Segino *et al.* and Migishima *et al.* showed that oocyte meiosis was unaffected[124] and fertility was retained[125]. Kagabu *et al.* had a major breakthrough when they found out that xenotransplantation of vitrified ovaries had reduced immunogenicity, mostly due to the osmotic dehydration that occurred[126].

6.3. How to preserve a sample

In order to preserve a biological sample at cryogenic temperatures, there are several things to be considered. Preservation techniques are a multi-step procedure that besides requiring proper technical parameters (optimal cooling and warming rates, for example), also try to balance key optimizations with cryoinjury mechanisms. Figure I.9 is a precise representation of the drawbacks that can be found during preservation and how one can proceed to counteract them.

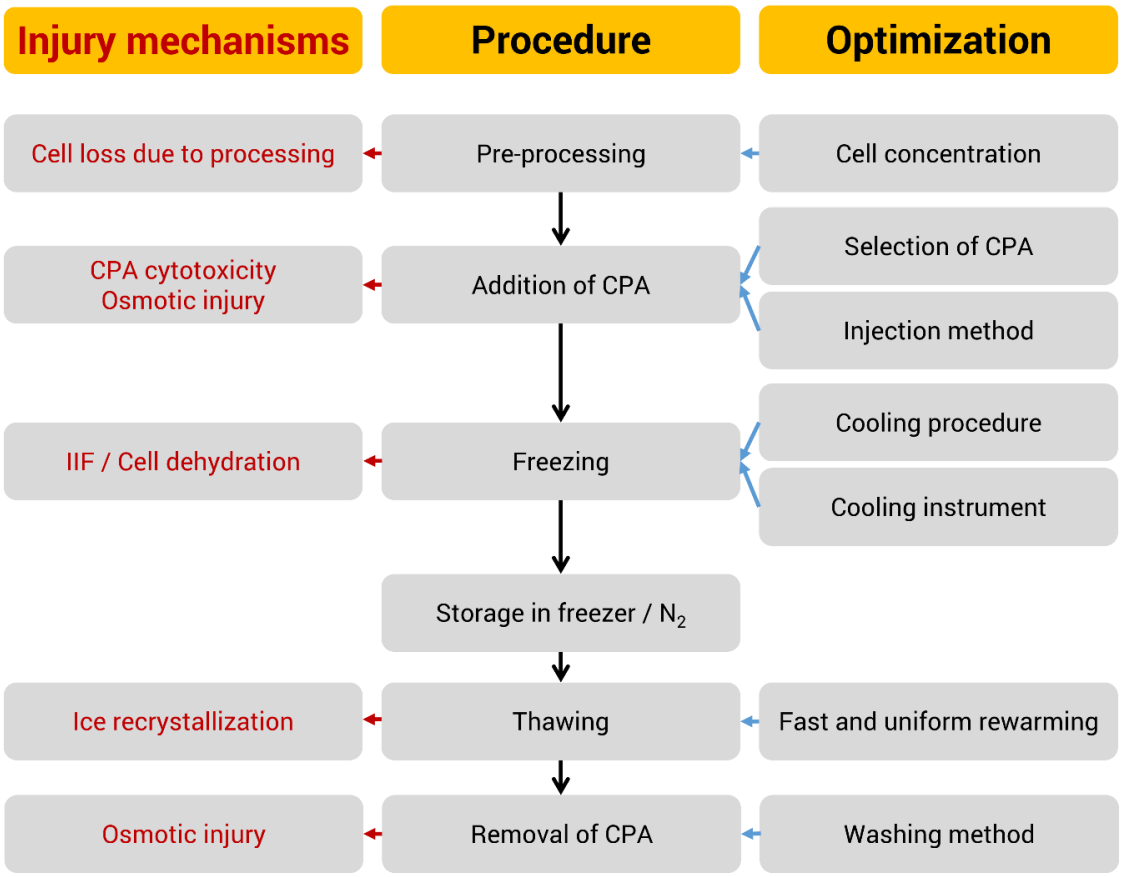


Figure I.9. Standard preservation procedure and associated difficulties. The items in the optimization column usually are regarded as research foci that contribute to the advance of preservation science.

In general, preserving a cell at cryogenic temperatures requires a CPA to be present, in order to avoid major cryoinjury. An equilibrium between concentration and cytotoxicity must be met for the CPA to be considered adequate. Then, both the cooling and warming parameters must be defined and fine-tuned to optimal survival. Because the water-to-ice phase change depends both on thermodynamic and kinetic processes, the cooling rate will define the extent of crystallization, which is why this is considered one of the most important factors.

Throughout the procedure, each step has an influence in cell volume. Therefore, the experimental setting must ensure that this variation is kept to a minimum to ensure maximal survival. Figure I.10 represents the cell volume at a given step of the cryopreservation procedure. In CPA addition, the cell volume will decrease because water will diffuse to the extracellular medium to hydrate the newly added solute, which will be co-diffused into the cell over time, resulting in a gradual volume increase.

Upon cooling, ice formation results in a reduction of the unfrozen water volume, which corresponds to a volume decrease. During storage, cell volume does not change due to kinetic hindrance but will progressively allow osmotic balance through the cellular membrane. Lastly, washing step is critical in cell survival: on one hand, cells become exposed to the CPA at physiological temperature, which results in increased toxicity if not removed; on the other hand, severe cell volume expansion occurs because solute removal is done by serial-dilution, which greatly increases the hypotonicity of the system.

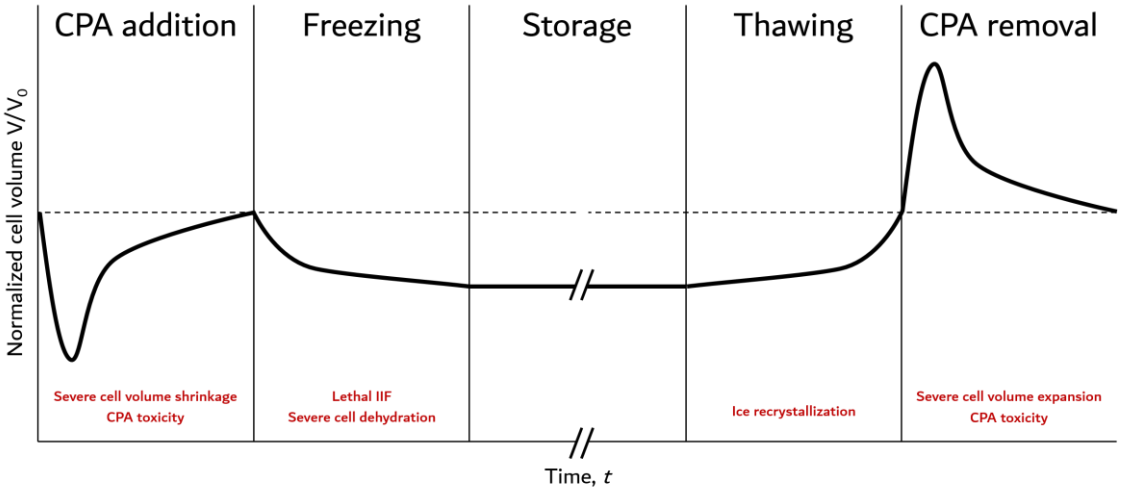


Figure I.10. Consequences associated with the step-wise procedure on volumetric transformations. Fine-tuning of a parameter to eliminate injury can usually exacerbate the nefarious effects on another stage.

6.3.1. Limitations of cell to organ scale-up

As mentioned, cryobiological research revolves around organ function. However, multicellular systems have two crucial limitations to standard cell techniques, and so far, strategies are still being thought out to avoid dysfunction.

Thermal heterogeneity is a technique-related issue. Whilst a given cooling rate is able to maintain cells at a given temperature, bulk tissues have more intricate heat transfer mechanisms. For instance, it has been shown that the cortical region of a kidney freezes earlier than the medullar region in its core, which rends a difficulty in homogenizing the procedure for a whole organ[127]. Mazur *et al.*[128] calculated that only slow cooling could be applied to kidney cryopreservation, despite the known effects of EIF. On thawing, thermal runaway is also unavoidable. This phenomenon suggests that upon inducing heat into a system, it accelerates exothermic metabolism that further increases temperature beyond what is desirable, resulting in nefarious damage.

Chemical heterogeneity is a diffusion-related issue. Organs are prone to total failure if only a partial region is undergoing dysfunction, so it is ideal that the CPA can disperse evenly throughout the whole volume. However, diffusion rates in compartments of different histology vary, and at a given time, different compartments might have different CPA concentrations[4]. If a heterogeneous distribution is present in a frozen organ, cryoprotection will vary and osmotic effects will be felt more aggressively in certain regions after thawing.

7. Bridging biotechnology with cryopreservation

In Section I.5.3, it became evident that polysaccharides can have a role in preservation. Biotechnology has had a major impact in providing scaled-up methodologies for preservation. The White House Organ Summit solicited that research now envisions batch-scalable “off-the-shelf” manufacturing instead of unscalable on-demand production[129].

In 2015, a great wave involving biotech companies started to develop emergent biomedical applications for both current needs and future warranty[1]. The bridge between biotech, biomedicine and cryopreservation has definitely been set when Kilbride *et al.* showed in 2016 that

a 2.3 L biomass of encapsulated liver spheroids that can be injected in bioartificial mimicking devices was fully cryopreserved[130]. The study demonstrated short-term viability and a 75.7% post-thaw viability was observed, but the key discovery was that the thermal heterogeneity issue was overcome in such a high volume of biomass (see Subsection I.6.3.1).

Biotechnology provides a means to obtain customizable polymers from natural sources that are biocompatible with the system of choice. The low temperatures implemented in cryopreservation also ensure that biodegradability is not an issue during storage, and only becomes convenient upon thawing, potentially reducing the need for a washout procedure.

7.1. Bio-based polysaccharides

Polysaccharides are high molecular weight molecules that contain 100 to 90,000 monomers bonded by a glycosidic linkage. While there are many sources of natural polymer production, bacteria are very attractive to work with due to their high growth and production rates. Also, the easily tailorable production conditions yield very diverse polymers regarding composition, size and structure. It was during the 19th century that an exopolysaccharide was first discovered in wine. Later, it became known as dextran and was found to be produced by the fermentative bacteria *Leuconostoc mesenteriodes*[131].

In general, polymers can be classified as intracellular storage polysaccharides (e.g. glycogen), capsular polysaccharides linked to the cell surface (e.g. antigens) or exopolysaccharides (EPS). Table I.4 shows the diversity of currently marketed biopolymers and their applications in several fields, and most of them are exopolysaccharides[132].

Table I.4. Characteristics of some bacterial exopolysaccharides used in commercial applications.

EPS	Source	Main monomers	MW	Properties	Applications	Ref.
Dextran	*Leuconostoc mesenteriodes*	Glucose	10^6 – 10^9	Non-ionic, stable, fluid behavior	Food, pharmaceutics, chromatography	133
Alginate	*Pseudomonas aeruginosa*	Guluronic acid Mannuronic acid	10^5 – 10^6	Film forming, gelling capacity	Food hydrocolloid, medical dressings, controlled drug release	133
Cellulose	*Acetobacter spp.*	Glucose	10^6	High tensile strength, insoluble in most solvents	Indigestible fiber wound healing, bioartificial blood vessels	134
Curdlan	*Myxogenes, Rhizobium, Agrobacterium radiobacter*	Glucose	10^4 – 10^6	Gel-forming, water insoluble	Petroleum industry, heavy metal removal	133
Xanthan	*Xanthomonas spp.*	Glucose, mannose, glucuronic acid	(2–50) x 10^6	Thermostable, chemostable, viscous	Pharmaceutics, cosmetics, personal care	133
Colanic acid	*E. coli, Shigella, Samonella and Enterobacter spp.*	Fucose, glucose, glucoronate, galactose	10^4 – 10^5	Gelling capacity	Cosmetics, personal care	133
Hyaluronan	*Streptococcus spp.*	Glucuronic acid, N-acetyl-glucosamine	(3–4) x 10^6	Cell adhesion and motility, wound healing, pseudoplastic and viscoelastic	Cosmetics, ophthalmic surgery, wound healing, surface coating	133

Bacteria-derived polysaccharides are a tempting resource in any field that requires polymer biocompatibility and freedom of customization to yield new, enhanced properties. Exopolysaccharides specifically, are widely used in several industries (namely the medical, pharmaceutical and food areas)[133] as some already have developed cheap and fast production strategies.

Exopolysaccharides are interesting molecules to implement in cryoprotective solutions for natural reasons. The purpose of its secretion mostly relates to functionality: the EPS matrix provides the backbone architecture of the biofilm in which bacterial colonies are able to thrive. On one hand, the biofilm mediates nutrient diffusion and cell-cell signaling. On the other hand, it protects bacteria from external physical and chemical stimuli, which is specifically important in preservation.

Back in 1981, Shrago *et al.* proposed that any substance with specific functional groups, namely –OH and –COOH terminals will have a cryoprotective effect, as they can participate in hydrogen bonding[135]. Some polysaccharides have shown to have intrinsic cryoprotective properties and Table I.5 summarizes some discoveries.

Table I.5. Collection of some literature results on polysaccharide use for cryopreservation. To a certain extent, it is plausible that any EPS should be able to provide a certain degree of cryoprotection.

Polymer source	Test cell line	Results	Ref.
Pseudoalteromonas arctica P-21653 7.4 x 10^6 Da Glucose, galactose	*E. coli*	The EPS successfully coats cells and increases cryoresistance: viability was 90% after the third freeze-thaw cycle (13% for saline control).	136
Colwellia psychrerythraea 34H Galacturonic acid-rich	---	The pseudohelicoidal structure prevents tetrahedral organization of water molecules in the first hydration shell, inhibiting ice growth. Ice inhibition is comparable to synthetic antifreeze proteins and its structure is similar to antifreeze glycoproteins.	137
Several pectic polysaccharides: Comuraman (*Comarum palustre*) Tanacetan (*Tanacetum vulgare*) Rauwolfia (*Rauwolfia serpentina*) Bergenan (*Bergenia classifolia*) Heracleum (*Heracleum sosnowskyi*)	*S. cerevisiae* Human leukocytes Human platelets Human erythrocytes	EPS potentiated glycerol preservation of leukocytes. Increased leukocyte viability and phagocytic activity. *S. cerevisiae* forms colonies after rewarming from -196ºC. Platelets had increased ability to aggregate. Platelets had greater hypotonic shock resistance. Erythrocyte hemolysis prevented, K^+ levels maintained.	138

7.2. FucoPol: a paradigm shift in preservation

In this work, a fucose-rich polysaccharide with a molecular weight of 1–5 MDa is the main focus in preservation research. FucoPol is an exopolysaccharide produced by *Enterobacter* A47 (DSM 23139), a gram-negative bacterium[139]. Its structure and composition are not fully elucidated, but Figure I.11 shows a proposed structure of FucoPol. It the composed of fucose (32–36 mol%), galactose (25–26 mol%), glucose (28–37 mol%), glucuronic acid (9–10 mol%) and about one-fourth as acyl groups, such as succinyl (2–3 mol%), pyruvyl (13–14 mol%) and acetyl (3-5 mol%)[140].

So far, research has shown that FucoPol is incredibly versatile for a variety of applications. Freitas *et al.* reported in 2011 the viscoelastic properties of FucoPol, comparable to guar gum and fucogel. Also, it demonstrated to be a good bioflocculant and emulsion stabilizer with potential in the food, pharmaceutical, cosmetic, textile, paper and petroleum industries[141].

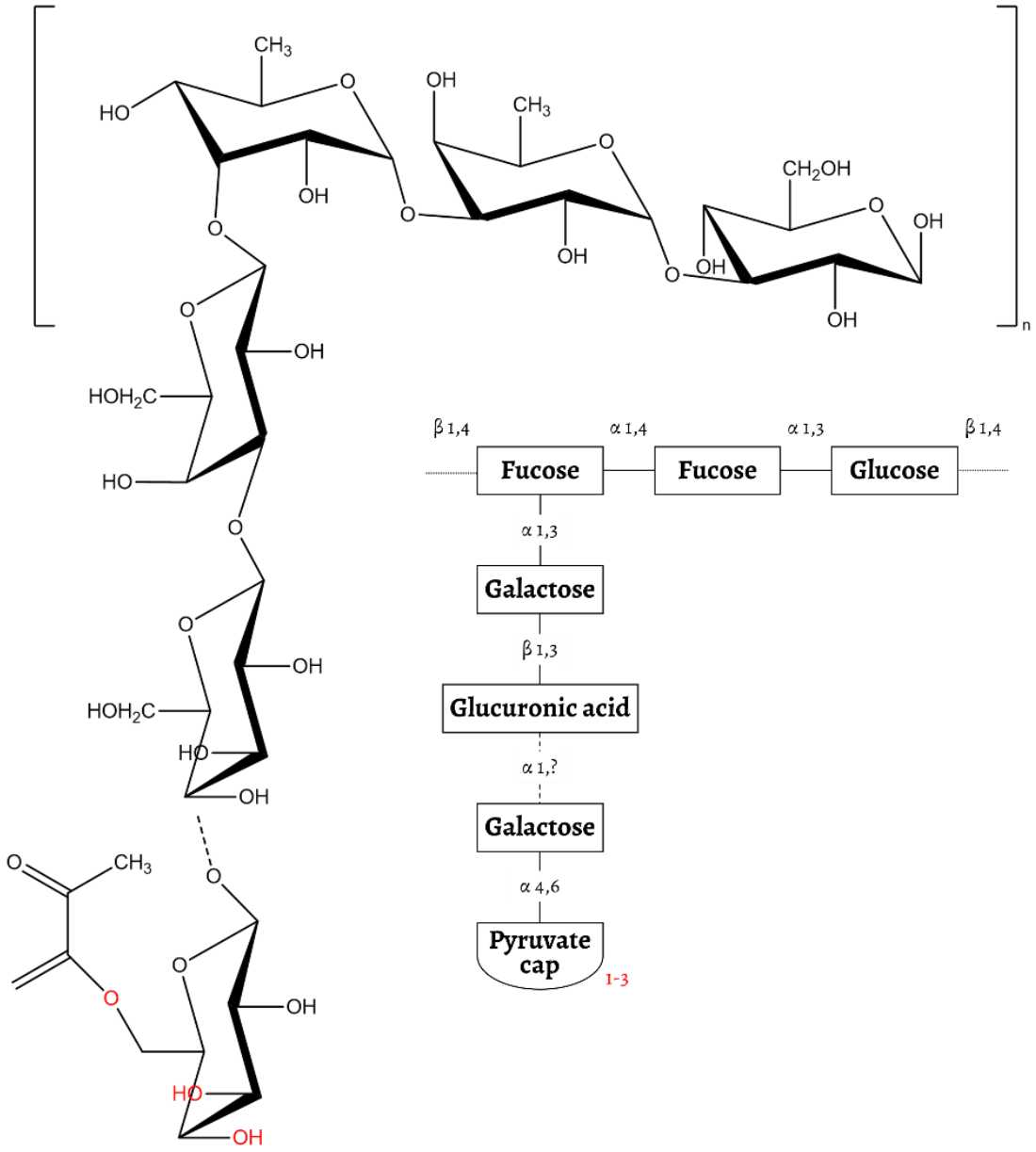


Figure I.11. Proposed structure of FucoPol based on preliminary experimental data. The structure presented is a deacetylated, desuccinylated form of the biopolymer. Its chain motif is composed of 1:1:2:1 galactose, glucose, fucose and glucuronic acid ratios. It contains 4 α and 1 β glycosidic linkages. Other monomers include 1.4:1:0.35 acetate, pyruvate and succinic acid ratios.

Ferreira *et al.* produced transparent, biodegradable FucoPol films with high hydrophilicity, permeability to water vapor and a good barrier to O_2/CO_2 diffusion[142]. Araújo *et al.* demonstrated the comparable adhesive properties of FucoPol to commercial glues and its role in the development of water-based glues suitable for different materials[143].

FucoPol has a hexamer motif that most likely is the repetitive oligomer of its structure. The (α, β) linkage intercalation induces structural bending, which probably yields a linear, helical shape with branching moieties capped by pyruvate terminals, also with helical bending. The structural periodicity of FucoPol is promising in crystal avoidance, as crystalline meshes also have periodical features that can be disrupted if the polymer is evenly distributed in the solution.

II. MOTIVATION

Although new formulas are being continuously being developed to answer specific demands, not much creativity is put into their composition besides the required categorical fulfillment described earlier. The Euro-Collins solution has been discarded because high glucose contents are disadvantageous for cell function, as it causes osmotic shock. Custodiol-HTK dominates the field of cardiac preservation but high volume perfusion (about 10 L used per organ) is frowned upon, requiring better cost-effective formulas. UW is currently the gold standard in liver, kidney and pancreas preservation[70] but its high viscosity, price and the fact that the antioxidant glutathione gradually oxidizes in the 1 L bags the formula is sold in are sound disadvantages that encourage the development of new formulas.

This work aims to thoroughly explore the properties of FucoPol as a bio-based polysaccharide and how adequate it can be for the development of novel cryoprotectant fluids. Although FucoPol was shown to have potential in several fields, we still lacked knowledge for its use in biomedicine, but some insights could be drawn from some rheological and thermochemical properties obtained by colleagues[144,145].

- ✓ **FucoPol presents a viscous, shear-thinning behavior**, which means that any applied pressure in fluid environments will decrease its dynamic viscosity. Blood is a new-Newtonian fluid that is under constant pressure of cardiac contraction, which yields its fluid-like features instead of a viscous substance that would result in vascular clogging. Thus, a shear-thinnable polymer is adequate for perfusion solutions as it can smoothly flow through the vasculature. It could also be used for immersion purposes as its viscosity will further protect the organ against external mechanical damage, very useful in accidental amputation cases or during transportation overall.

- ✓ **FucoPol maintains a characteristic shear-thinning curve even under temperature variations.** Also, when subjected to iterative heating and cooling cycles, it did not accumulate any thermal history, which means its structure remained intact and the shear-thinning behavior is preserved. Thus, FucoPol is able to tolerate the freeze-thaw cycles used in cell and organ preservation without compromising its function by some form of structural degradation.

- ✓ **FucoPol maintains its shear-thinning behavior under extreme pH and ionic strength conditions.** This further complements its potential use in cryobiology for two reasons: (i) the stability of FucoPol means it can be tailored to fit nutrient-specific media, a key criterion in formula development research; and (ii) the ability of FucoPol to preserve its function in extreme pH and ionic strength values is essential in the case of acidosis or abrupt tonicity changes, respectively.

The challenge of CPA toxicity also remains unsolved and a big issue in biological preservation but Clark *et al.* showed that dextrose can prevent the irreversible binding of DMSO to proteins crucial to cellular function, thereby resulting in toxicity reduction[84]. As dextrose is a sugar, polysaccharides like FucoPol might be able to intrinsically neutralize the cytotoxicity of DMSO or at least reduce it to levels that still support DMSO use in biological applications.

III. MATERIALS AND METHODS

1. FucoPol production

1.1. Microorganism

Enterobacter A47 (DSM 23139) is a gram-negative, facultatively anaerobic, coliform bacteria of the Enterobacteriaceae family. It was preserved in glycerol (20 %, v/v) as a cryoprotectant agent, at -80ºC.

1.2. Media

Solid medium for bacterial plating was prepared by weighing 9.9 g of Chromagar Orientation, a nutrient-rich formula composed of Luria-Bertani (LB) medium (for 1 L: 10 g tryptone, 10 g NaCl and 5 g yeast extract), agar and chromatic substances and dissolving in 300 mL of distilled water in a Schott flask. The solution was sterilized by autoclaving at 121 ºC for 20 min. In a laminar flow chamber, while still hot, the medium was used to half-fill Petri dishes, which were left to cool to room-temperature. The dishes were then closed, sealed with parafilm, wrapped in aluminum foil and stored at 4 ºC.

Liquid pre-inoculum: is the medium in which the bacterial cells will grow to a considerable amount before being transferred to the liquid inoculum. In a 100 mL Erlenmeyer flask, 20 mL of Luria-Bertani (LB) medium were poured and agitated. To prepare liquid LB medium, 1.0 g/L peptone, 0.5 g/L yeast extract and 1.0 g/L NaCl (pH 6.8-7.0) were added. The solution was sterilized by autoclaving at 121ºC for 20 min.

Liquid inoculum (medium E*) is the acellular medium to which *Enterobacter* A47 will be transferred to before the bioreactor cultivation. In a 500 mL Erlenmeyer flask, 1.16 g K_2HPO_4, 0.74 g KH_2PO_4, 0.66 g $(NH_4)_2HPO_4$ were dissolved in 200 mL of distilled water. The solution was sterilized by autoclaving at 121ºC for 20 minutes.

Liquid bioreactor medium (medium E*) is identical to the liquid inoculum, but scaled up in volume. To a 1 L Erlenmeyer flask, 9.86 g K_2HPO_4, 6.29 g KH_2PO_4 and 5.61 g $(NH_4)_2HPO_4$ were added to distilled water for a final volume of 1 L. The full content was transferred to the bioreactor chamber and an extra 700 mL of distilled water were added, for a final working volume of 1.7 L. The solution was sterilized by autoclaving at 121ºC for 20 minutes.

Liquid supplementation set is the secondary collection of nutrients that supplement medium E* but are autoclaved separately to avoid salt precipitation:

- To prepare an $MgSO_4$ solution, 25.0 g of $MgSO_4.7H_2O$ in 1 L of distilled water. This solution was then fractioned: for the inoculum medium, 5 mL were added to a Falcon tube; for the bioreactor medium, 20 mL were added to another Falcon tube. Both tubes were sterilized by autoclaving at 121ºC for 20 min and stored at 4ºC.

- For the mineral solution, 82 mL of HCl were diluted in 920 mL of distilled water to prepare 1 L of a 1 N HCl solution to be used as solvent. Then, 2.78 g $FeSO_4.7H_2O$, 1.98 g $MnCl_2.4H_2O$, 2.81 g $CoSO_4.7H_2O$, 1.67 g $CaCl_2.2H_2O$, 0.17 g $CuCl_2.2H_2O$ and 0.29 g $ZnSO_4.7H_2O$ were added to the acidic solvent and stored at 4ºC in a dark bottle. For usage, this stock solution must

first be diluted 10-fold in distilled water. Then, the medium was fractioned: for the inoculum medium, 5 mL were added to a Falcon tube; for the bioreactor medium, 20 mL were added to another Falcon tube.

- For the glycerol solution, 100 mL were poured to a 200 mL Schott flask to supplement the inoculum. For the bioreactor medium, 80 mL were poured to a 100 mL Schott flask. Both tubes were sterilized by autoclaving at 121ºC for 20 min and stored at room-temperature.

Feeding solution is the nutrient input performed while the bioreactor is active. It is composed of medium E* supplemented with glycerol at a concentration of 200 g/L. The solution was sterilized by autoclaving at 121ºC for 20 minutes.

1.3. *Enterobacter* A47 reactivation

Reactivation of *Enterobacter* A47 was done by scraping the surface of the cryovial containing the microorganism and scratching a Chromagar plate. The plate was incubated at 30ºC for 24 h until isolated colonies could be pinpointed.

1.4. Inoculum preparation

The pre-inoculum was prepared by inoculating a single colony isolated from the Chromagar Orientation plate into 20 mL LB medium (in a 100 mL) shake flask, and incubation in an orbital shaker, at 30ºC and 200 rpm for 24 h. To prepare the inoculum, the pre-inoculum (20 mL) was transferred into 200 mL Medium E*, supplemented with 40 g/L glycerol (in a 500 mL baffled shake flask), and incubated at 30 ºC for 60-70h under 200 rpm continuous agitation in an orbital shaker.

1.5. Bioreactor cultivation

A bioreactor is a bioengineered vessel that supports a biologically active environment and allows the control of input and output dynamics. For FucoPol production, a 2 L bioreactor was used and controlled by a Sartorius Biostat B plus automation device that defines the parametric set-points for the experiment. Figure III.1 displays the built-in experimental setting, although its disposition is arbitrary for correct function.

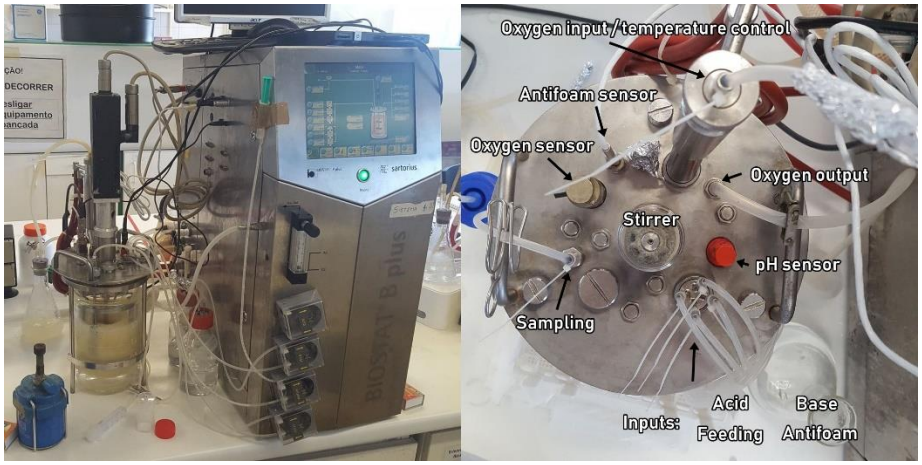


Figure III.1. Experimental setup of the bioreactor procedure. The bioreactor piece (right) contains specific components to mediate internal conditions, which are regulated by the core component (left). The assembly of the inputs and ouputs is arbitrary.

To the bioreactor main steel piece, all parts, pins and pipes were connected and covered with cotton and aluminum foil and autoclaved for 30 min at 121ºC. The dO_2 sensor was calibrated to be in a range of 15–200 nA, and left at 70 nA. The pH sensor was calibrated and its set-point defined to 7.00 ± 0.05. Stirring was set to 300–800 rpm to lock dO_2 = 10% at all times. The temperature set-point was gradually increased from 26ºC to 30º, for a final set-point of 30 ± 0.1ºC. Aeration rate was set to 3 SLPM (standard liters per minute).

The bioreactor chamber was loaded with 1.7 L of liquid medium E*. Subsequently, with the aid of a Bunsen burner in the near proximity of the input hole (for sterility purposes), 20 mL $MgSO_4$, 20 mL mineral solution, 80 mL glycerol and 220 mL of the inoculum were added.

The sampling procedure was done over a course of 3 days, or 75 h specifically. After 8 h of initiating the bioreactor, the feeding medium was automatically supplied at 11 $g/L{\cdot}h^{-1}$ to the chamber.

In day 1, sample #0 was nominated the reference sample for the growth stage. Then, 5 mL samples were withdrawn from the bioreactor chamber each hour, and 23 mL samples every 4 h. The **5 mL samples** were used to measure the A_{450} (see Section III.8.1). The hourly withdrawal was only done in the first 8 h of experiment to ensure bioreactor welfare and trace a sufficiently well-defined growth curve. The **23 mL samples** were used to measure the A_{450} and the apparent viscosity, and to quantify the biomass, nutrients and FucoPol. The samples were centrifuged at 15777 *g* for 15 min at 4ºC. Viscous samples were diluted prior to the centrifugation. The cellular pellet was discarded and the supernatant containing FucoPol was stored at -20ºC for further analyses. The 4-hourly withdrawal was only done twice in the first 8 h of experiment. For the following days, a sample was withdrawn 3 times per day with a 3-hour interval.

After 75 h of cultivation, the bioreactor experiment was stopped and the broth was collected into a 5 L Schott flask that was kept at 4ºC.

1.6. Analytical techniques

Apparent viscosity was determined in a Fungilab alpha viscosimeter by using an LCP pendulum to apply rotational shear to 17 mL samples. The viscometer used has different RPM velocities for its steel pendulum: a greater broth viscosity requires lower RPM values to obtain a trustworthy result (between 10 and 90% as shown in the equipment display). Thus, different RPM were used (in descending order) to obtain dynamic viscosities, which implies a normalization of all values to a single RPM value (shear rate).

For further calculation, a linear approximation was used, given that 1 RPM = 1.2236 mPa for the LCP pendulum used[146], and the results shown in Table IV.1 are already corrected. However, bear in mind that FucoPol has a shear-thinning behavior, which is a non-linear tendency. This means that linear approximations (representing Newtonian fluids only) inaccurately reflect dynamic viscosities at higher values, though this discrepancy was neglected as higher values have a lower error impact on the expression of true value.

High-pressure liquid chromatography (HPLC) was used to assess glycerol concentration over time. The HPLC equipment is a versatile technique for analytical separation of the constituents in a mixture and single identification. In general, a pressurized liquid solvent is run through a column of milimetric diameter that contains a solid adsorbent material, such as silica or a physisorbed polymer of a given size. The interaction of the constituents in a mixture with the adsorbed material will result in their elution at different slow rates, enabling their differentiation. A ELITE LaChrom

HPLC (column oven: VWR L-2350, autosampler: VWR L-2200, pump: VWR L-2130, detector: MERCK RI-71) was used in this work to evaluate the carbon content of the system after bioreactor production. The HPLC run was done at 50ºC during 18 minutes with an injection rate of 0.6 mL/min for 99 µL injection samples.

The cell-free supernatant samples diluted 50-fold in the eluent, H_2SO_4 0.01 N, and 800 µL of the resulting solution were filtered in Corning® Costar® Spin-X® Plastic centrifuge eppendorf filters. The filtrate was transferred to an HPLC vial containing a puncturable sept. To obtain a calibration pattern, a 1.3 g/L glycerol solution was prepared from a 99% w/v. Then, four successive 2-fold dilutions were performed with distilled water to yield five reference solutions of 1.30, 0.65, 0.33, 0.16 and 0.08 g/L glycerol. These were equally filtered and stored in HPLC vials.

Continuous flow analysis (CFA) was used to assess ammonia concentration over time. The Skalar SAN^{++} System equipment is a continuous flow analyzer used in environmental applications for the measurement of analytes such as ammonia, nitrite, phosphate and other ions in the food and agricultural industries and can be reliable in quality control. In general, the sample of interest is injected into a flowing carrier solution that contains a reactive reagent that loops in narrow tubes. In contact with the substance of interest, the samples develops a color that determines its concentration in the sample. After FucoPol production, the ammonia concentration in each sample collected was determined by a modified Berthelot reaction (chlorination of ammonia to react with salicylate, finally yielding an oxidized 5-aminosalicylate complex of green color measured at 660 nm) by simply injecting the samples into the flowing system.

Sample preparation was identical to the way it was performed for HPLC, except the dilution step is performed 200-fold with distilled water containing 50 ppm of sodium azide, a bactericide used to guarantee sterility and avoid polymer loss by consumption. Samples were then transferred to 4 mL sample tubes. Calibration was done by preparing solutions of 2, 4, 8, 16 and 20 ppm NH_4Cl.

Dialysis was done to the bioreactor samples collected over time. Supernatant samples (5 mL) were dialyzed in distilled water using ROTH ZelluTrans ϕ 28.6 mm dialysis strips at room-temperature, until solvent conductivity < 20 µS/cm. Between each solvent exchange, 50 ppm of sodium azide were added. After dialysis, the strip contents were transferred to pre-weighed tubes, which were lyophilized at -100ºC (SCANVAC CoolSafe). The resulting dry polysaccharide was collected and weighed to assess production parameters.

1.7. Extraction and purification

The final 1.2 L volume of bioreactor broth was first diluted 5-fold with distilled water. To remove cellular impurities before dia/ultrafiltration, centrifugation (Sigma 4-16KS) was performed at 10375 *g* for 45 min at 4ºC. After the first centrifugation step, the supernatant was collected to heat-resistant Erlenmeyer flasks and left to incubate at 70ºC for 1 h to denature any proteins. Then, the supernatant was re-centrifuged and jointly stored at 4ºC in a 10 L Schott flask.

Dia/ultrafiltration was performed with a Sartorius 100 kDa membrane mounted on a vertical device. The procedure was done in a cross-flow fashion, was continuous and hourly samples of the 10 L Schott flask were collected and returned until conductivity < 20 µS/cm. For specific details on the set-up, refer to Figure III.2. The broth was then concentrated to about 1.5 L (by shutting down the water supply) and stored at 4ºC.

The solution was frozen to -80ºC and then lyophilized at -100ºC. The resulting dry polysaccharide was collected, weighed and used for all subsequent experiments.

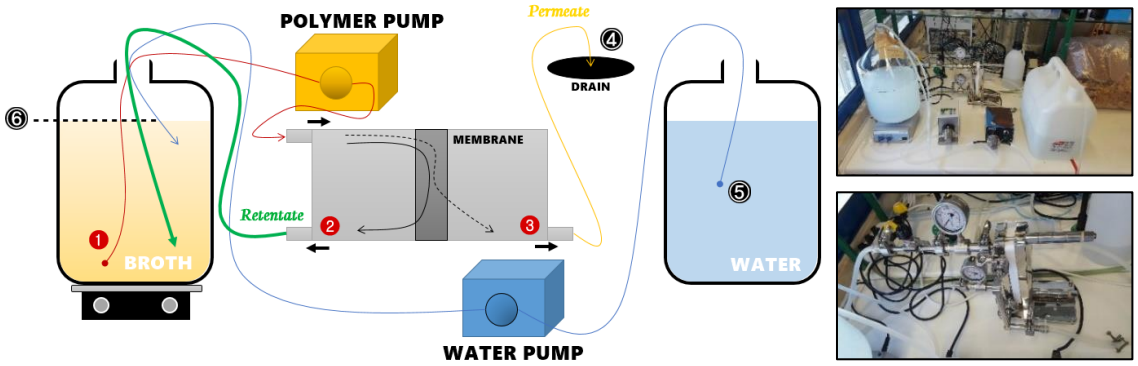


Figure III.2. Schematic representation of the dia/ultrafiltration device. The treated cell-free supernatant is pumped into the membrane in diafiltration mode (1): particles bigger than the cut-off molecular weight of the membrane (100 kDa) like FucoPol will be retained and returned to the broth (2), while the solvent and smaller particles will permeate the membrane and be expelled to the drain (3). The broth becomes concentrated but permeation results in volume loss, which will increase pressure at the polymer pump (4). Therefore, a simultaneous process (ultrafiltration mode) injects water (5) through a similar pump directly into the 10 L Schott flask to maintain the broth level (6). In the top-right and bottom-right are pictures of the setup used and a close-up of the membrane device, respectively.

2. Physicochemical properties

2.1. Solution preparation

To assess the physicochemical properties of FucoPol and other solutions in its presence, 3 solutions and 2 mixtures were prepared.

To prepare 0.5% w/v FucoPol, 0.4 g of the polymer were dissolved in distilled water with magnetic stirring for 2 h. Keeping the agitation process at 50ºC aids in the dissolution but higher temperatures may produce a rubbery, paste-like solution: as such, slow agitation but at room-temperature is advised.

To prepare 0.5 mg/mL bovine serum albumin (BSA), 5 mg were scattered on the surface of 10 mL of distilled water in a sample tube and left to dissolve at 4ºC for *ca*. 30 min to an hour.

To prepare 2X Euro-Collins, a cryoprotective solution, an ordered addition of 8.64 g D-glucose (anhydrous), 39.4 mg $MgSO_4.7H_2O$, 0.20 g $KHCO_3$, 0.80 g KH_2PO_4, 1.01 g K_2HPO_4, 116.8 mg NaCl and 74.6 mg KCl was performed and dissolved in 100 mL of distilled water with continuous magnetic stirring. The pH was corrected to 7.0.

To prepare a mixture of FucoPol and BSA, 10 mL of the 0.5% w/v FucoPol solution were co-diluted with 10 mL of the 0.5 mg/mL BSA solution, to yield a final concentration of 0.25% FucoPol and 0.25 mg/mL BSA. The mixture was left to homogenize in slow stirring for 10 min.

To prepare a mixture of FucoPol and Euro-Collins, 10 mL of the 0.5% w/v FucoPol solution were co-diluted with 10 mL of the 2X Euro-Collins solution, to yield a final concentration of 0.25% FucoPol and 1X Euro-Collins. The mixture was left to homogenize in slow stirring for 10 min.

2.2. Assessment of macroscopic and microscopic traits

To determine the properties of both the individual substances and mixtures, macroscopic (color, precipitate, homogeneity) and microscopic traits (pH, conductivity, dynamic viscosity) were determined.

For the macroscopic properties, color was determined visually and arbitrarily nominated by visual comparison. Presence of any precipitate was assessed by inverting the recipient and visualizing its base. Homogeneity was determined as a measure of absence of any color gradients or fluctuating smears in the solution.

For the microscopic properties, pH was assessed with a VWR pHenomenal pH 1100L pH/mV meter by immersing a glass electrode on *ca*. 4 mL of solution. Conductivity was assessed with a BANTE Instruments 510 conductivity/TDS meter by immersing a metal electrode on *ca*. 4 mL of solution. Dynamic viscosity was assessed with a Fungilab alpha viscosimeter using 17 mL of solution in the sample holder and an LCP spindle.

Given the qualitative approach employed, single measurements at equilibrium were made for pH and conductivity, and triple measurements for dynamic viscosity at 2.5, 10 and 12 rpm, once each. For the conversion of rpm to a constant shear rate to obtain a representative value of dynamic viscosity, refer to Section III.1.6.

3. Antifreeze properties

3.1. Flash freezing assay

To evaluate the ice-inhibiting properties of FucoPol in a quick way, eight solutions were prepared in small volumes to be placed inside the tip of a pipette. Solutions of 1, 0.5, 0.25, 0.13 and 0.06% w/v FucoPol were prepared and contrasted against water as the blank reference and 50% v/v glycerol as the positive control.

Upon loading of the solutions onto a manually built multi-pipette device, all tips were simultaneously immersed in liquid N_2 (-196ºC). After 10 s, the device was removed from the flash freezing recipient and the solution volume was visually interpreted according to the presence (opaque solution) or absence of ice (translucent solution). For the assay schematics, refer to Section IV.3.1 as it provides visual aid in the discussion.

3.2. Differential Scanning Calorimetry profiling

Thermodynamic profiling of several liquid samples was performed to assess the relative antifreeze potential of FucoPol. The DSC instrument is a thermoanalytical tool that can characterize the direct behavior of solid powders or the indirect influence a dissolved constituent can have in a solvent when a temperature change occurs. By implementing heating and cooling cycles, the DSC technique yields the thermodynamical events the system undergoes, like crystallization and melting, which are represented by exothermic and endothermic enthalpies, respectively, and an onset temperature. Secondary events like glass transitions, cold crystallization, thermal aging and heat capacity variations can also be observed and give information on sample behavior. While the melting event is solely dependent on thermodynamic conditions, crystallization is influenced both by a thermodynamic and a kinetic effect, which means different cooling rates will yield different results, while not necessarily observing different phenomena.

In Figure IV.5, a loop is observable in the crystallization event. Exothermic events usually present as normal peaks and its appearance is related to the specific equipment used and is not an erroneous behavior. While the sample was cooling by injection of liquid N_2 to the closed device chamber, the exothermic transition of water to ice released such thermal energy that it transiently increased the temperature of the chamber, as detected by the sensor, which explains the loop-like feature[147]. Enthalpy values are collected by peak integration, which for the crystallization loop, can be done if the x-axis is converted from temperature to time, yielding an energy value but independent of temperature variations.

To assess normal water behavior, distilled water was used as a blank reference. The reference samples for behavior profiling were BSA, a non-antifreeze protein, and HPLC6, a type I antifreeze protein from *Pleuronectes americanus* obtained from A/F Protein Inc (refer to Table III.1 below). All solutions were syringed to TA Instruments Tzero Aluminum Hermetic capsules and targeted to have an average mass of 20 mg. However, priority was given to fully filling the basal surface of the capsule and levelling the meniscus. The loaded capsules were then tight-sealed and placed in the equipment for thermal analysis. In a TA Instruments Q2000 DSC coupled with a TA Instruments Refrigerated Cooling System 90, each sample was submitted to a 2-cycle thermal treatment. At a constant rate of 10ºC/min, the sample was equilibrated at 40ºC, cooled to -90ºC, heated to 80ºC, cooled to -90ºC and heated to 40ºC. At every temperature inflection of the cycle, the sample was allowed to equilibrate for 1 min.

Table III.1. Summative table of the solution preparation for DSC thermal analysis. The sample name is determined by its identifier, which will be used throughout the thesis. Sample mass was determined by subtracting the capsule weight. The mass ratio is the quotient between the mass before analysis and the mass after analysis (higher values indicate greater evaporation).

Identifier	Sample mass	Mass ratio	Description
dH_2O	30.84 mg	2.04	Distilled water (18.22 µS/cm)
BSA 1	25.78 mg	1.11	100 mg of BSA were dissolved in 100 mL of distilled water for a final concentration of 1 mg/mL
AFP 1	43.05 mg	1.40	100 mg of AFP were dissolved in 100 mL of distilled water for a final concentration of 1 mg/mL
FP 10	33.40 mg	1.52	30 mg of BSA were dissolved in 3 mL of distilled water for a final concentration of 10 mg/mL
FP 1	25.11 mg	1.13	The FP10 solution was diluted 10-fold in distilled water to obtain a final concentration of 1 mg/mL
FP 0.1	21.34 mg	1.38	The FP1 solution was diluted 10-fold in distilled water to obtain a final concentration of 1 mg/mL
FP-BSA 1:10	23.02 mg	1.11	0.1 mg of FucoPol and 1 mg of BSA were co-dissolved in 1 mL of distilled water for a final concentration of 0.1 mg/mL FucoPol + 1 mg/mL BSA
FP-BSA 1:1	22.80 mg	1.12	1 mg of FucoPol and 1 mg of BSA were co-dissolved in 1 mL of distilled water for a final concentration of 1 mg/mL FucoPol + 1 mg/mL BSA
FP-BSA 10:1	23.10 mg	1.10	10 mg of FucoPol and 1 mg of BSA were co-dissolved in 1 mL of distilled water for a final concentration of 10 mg/mL FucoPol + 1 mg/mL BSA
FP-AFP 1:10	26.11 mg	1.14	0.1 mg of FucoPol and 1 mg of AFP were co-dissolved in 1 mL of distilled water for a final concentration of 0.1 mg/mL FucoPol + 1 mg/mL AFP
FP-AFP 1:1	23.48 mg	1.07	1 mg of FucoPol and 1 mg of AFP were co-dissolved in 1 mL of distilled water for a final concentration of 1 mg/mL FucoPol + 1 mg/mL AFP
FP-AFP 10:1	38.60 mg	1.24	10 mg of FucoPol and 1 mg of AFP were co-dissolved in 1 mL of distilled water for a final concentration of 10 mg/mL FucoPol + 1 mg/mL AFP

All capsules were punctured to avoid pressurization and potential burst of the capsule due to evaporation. To account for mass loss and adjust enthalpy values, the capsules were weighed both before and after the thermal analysis. Table III.1 summarizes the method of preparation of all solutions tested, final concentrations and each individual mass data.

4. Cryopreservation assays

4.1. Sterilization

To implement FucoPol in *in vitro* assays, several sterilization methods were tested. For dry-state autoclaving (method 1), 0.12 g of FucoPol were weighed and inserted in a Schott flask. The flask was fully sealed to avoid any water humidifying the polysaccharide, and then it was autoclaved at 121ºC for 20 min.

For liquid-state autoclaving (method 2), a 0.25% w/v FucoPol solution was prepared in a Schott flask and autoclaved at 121ºC for 20 min.

For alcohol-mediated asepsis (method 3), 0.12 g of FucoPol were weighed was mixed in 50 mL of 70% v/v ethanol, for a final concentration of 0.25% w/v FucoPol. The suspension was then poured into a Petri dish, covered with punctured aluminum foil in a laminar flow chamber and left to fully evaporate.

For UV irradiation (method 4), a 0.25% w/v FucoPol solution in distilled water was either prepared in a glass flask (4.1) or a plastic tube (4.2). Then, the solution was irradiated in a UVP CL-1000 Ultraviolet Crosslinker at 254 nm for 2 h.

By filtration (method 5), a 0.25% w/v FucoPol solution in distilled water was prepared and loaded onto a syringe and transferred to a sterile Schott flask through a 0.2 µm GE Healthcare Life Sciences Whatman™ ME 24/21 ST attachable syringe filter, in a laminar flow chamber.

After performing all procedures, all samples were either dissolved in or diluted 2-fold with resazurin, a metabolic indicator that signals the presence of any live microorganism, and left to incubate at 37ºC for 2 h. To understand the mechanism of action of resazurin, refer to Section III.8.3. The full sterilization diagram is alternatively presented in Section IV.4.1 as it provides a visual aid for discussion.

4.2. Cytotoxicity assay

To assess if FucoPol was potentially cytotoxic to Vero cells, a cytotoxicity assay was performed. For a detailed description of the Vero cell line, refer to Table III.3. A stock solution of 1% w/v FucoPol was prepared in culture medium and left to agitate in an orbital shaker at room-temperature overnight. Then, five successive 2-fold dilutions were performed with culture medium, yielding 1, 0.5, 0.25, 0.13, 0.06 and 0.03% w/v FucoPol for a total of six test conditions. After 24 h of incubation at 37ºC, 5% CO_2, the adhered Vero cells were exposed to 100 µL of each FucoPol solution, DMEM as negative control and 10% DMSO as positive control.

The detailed procedure is identical to what is presented in Figure III.3, with a few exceptions: (i) the freezing section was not performed as cytotoxicity assays are done at normothermic conditions and as a result, 10% DMSO was not added to the total mix, (ii) the amount of seeded cells was

6,000/well and (iii) an additional 24 h incubation period at 37ºC, 5% CO_2 in DMEM was performed before exposing the adherent cells to the test solutions.

4.3. The *in vitro* cryopreservation standard procedure

To initiate a cell culture procedure, the cell line (*e.g.* Vero) was collected from the -80ºC freezer and quickly thawed in a 25ºC water bath until only *ca.* 10% of visible ice is present. Then, 1 mL of cells were pipetted to a T-25 culture flask in a laminar flow chamber, along with 4 mL of cell culture medium (*e.g.* Dulbecco's Modified Eagle Medium (DMEM)). The T-flask was left to incubate at 37ºC, 5% CO_2 until about *ca.* 70% cell confluency was attained by periodically checking in an optical microscope.

Cell detachment: When a sufficient amount of cells was present to perform the experiment, the culture medium was aspirated from the T-flask and the cells were washed with Ca^{2+}-free, Mg^{2+}-free (trypsin inhibitors) Phosphate Buffer Saline (PBS), which was gently shaken and aspirated once again. To detach the cells, 500 µL of Gibco® TrypLE™ Express trypsin were added and the T-flask was left to incubate at 37ºC, 5% CO_2 for 10 min. After observing adequate detachment (aggregates of 3 cells or less) in an optical microscope, 2 mL of culture medium were added and the cells were gently resuspended inside the pipette *ca.* 10 times to avoid foam formation. The cell suspension was then transferred to a Falcon tube for cell count.

Cell count: From the Falcon tube, 100 µL of cell suspension were withdrawn and mixed with 100 µL of trypan blue (a permeant colorimetric indicator) by pipette resuspension. To prepare the counting device, a Hirschmann EM Techcolor Neubauer Improved hemocytometer was coupled with a sticking lamella that was humidified in the edges with isopropanol. A sufficient volume of the cells-trypan mix was gently pipetted to the counting chambers of the hemocymeter in a way that it fills the whole square by capillarity. For specifications on cell counting, refer to Section III.8.2.

Cell seeding: After cell counting, the cell suspension was centrifuged at 100 *g* for 5 min and resuspended with an adequate volume corresponding to a concentration of 200,000 cells/mL. Then, a 96-well microplate (in duplicate) was loaded with the cell suspension, 10% DMSO and the test solutions specific for each experiment, for a total volume of 50 µL containing 20,000 cells/well. Upon loading, the basal diameter of the well must be fully submersed. Bubble formation was avoided by slowly pipetting inside the liquid instead of circularly shaking the microplate, as this will provide centrifugal force and cells will adhere to the wall.

Freeze-thaw cycle: After preparing both microplates for screening the test systems, the control microplate (RT) is left to incubate at 37ºC, 5% CO_2 for 30 min. The test microplate (CRYO) was sealed with parafilm and immediately frozen to -80ºC inside a styrofoam recipient designed to yield an optimal cooling rate of 1ºC/min. After 24h, CRYO was thawed in a 37ªC temperature-controlled water bath until only 10% of visible ice is present, but never surpassing the 2-minute mark. In a laminar flow chamber, the contents of the wells were diluted 6-fold with culture medium and left to incubate at 37ºC, 5% CO_2 for 24 h for cells to regain normal metabolic rate. This procedure is designed to assay cells in suspension, as this system better mimics the physiological environment and reduces technique-related negative effects upon freezing. After the 24-hour incubation, cells already adhered but do not skew the viability results.

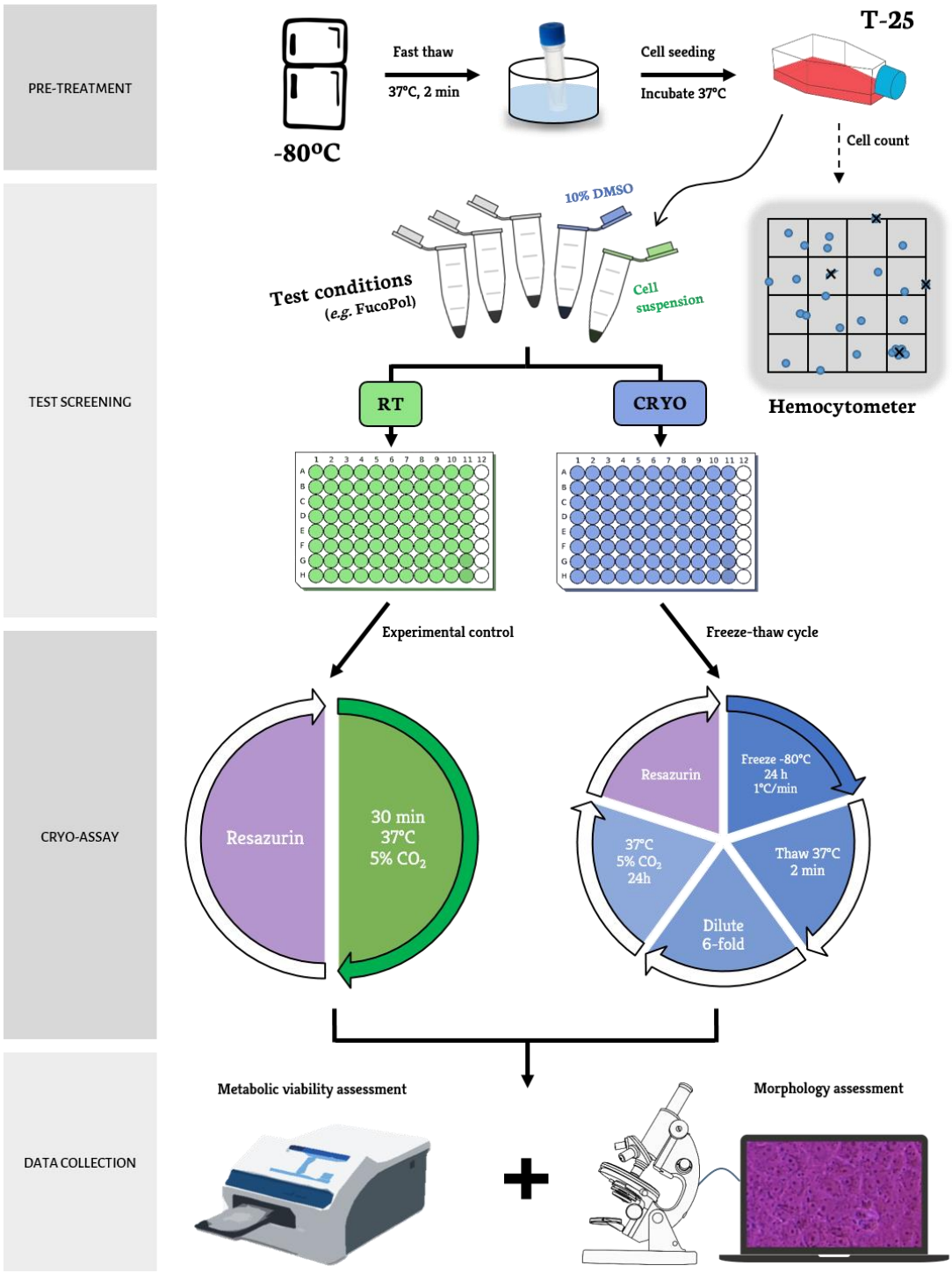


Figure III.3. Schematic representation of the full treatment necessary to perform a suspended cell cryo-assay. The cell culture is unfrozen for use, quantified, exposed to the test substances and then frozen under their presence to assess their cryoprotective potential. After thawing, both functional and structural viabilities are assessed. This procedure was optimized by trial and error.

Cell viability assessment: To assess cell viability, the metabolic indicator resazurin was used. For a detailed explanation on the mode of action of resazurin, refer to Section III.8.3. To RT, 250 µL of 60% resazurin diluted in culture medium were added to each well (6-fold dilution) and left to incubate at 37ºC, 5% CO_2 for 4 h. To CRYO, after discarding the liquid contents of each well, 100 µL of 50% resazurin diluted in culture medium were added to each well and left to incubate at

37ºC, 5% CO_2 for 2 h. After incubation times, absorbance was measured at 570 and 600 nm in a BioTek® microplate reader and visualized in the BioTek® Gen 5 software. Resazurin in the absence of cells was used as the reference value. Culture medium in the presence of cells as a test condition was used as the positive control.

Optical microscopy image collection: to assess the morphological characteristics of cells in the presence or absence of FucoPol, a NIKON Eclipse Ti optical microscope connected to a NIKON digital camera was used. The liquid contents of all wells were discarded and pictures were collected in the Camera Control Pro software, with the following technical specifications: 10x objective lens, shutter priority exposure mode, 1/10 s shutter speed, 400 ISO sensitivity, JPEG fine image quality with optimal compression, 3936x2624 image size, 14-bit NEF bit depth, 800mm lens with f/13G aperture and sRGB color space. Image regions are arbitrary selections of the representative phenomena observed and may only partially reflect the full state of a cellular system.

4.4. Normothermic formula preparation

To assess the cryogenic performance and stability of FucoPol in different ionic environments, a total of 7 normothermic formulas were assayed, of which 5 were prepared from scratch. In practical terms, 14 conditions were tested, both in the presence and absence of 0.25% w/v FucoPol, following the protocol detailed in Figure III.3. Preparation involved simple ordered dissolutions of all salts to a final volume of 1 L in distilled water and storage at 4ºC. All solutions were deemed viable for use for a maximum period of 1 month, upon which a fresh solution was prepared again. To prepare the test media supplemented with 0.25% w/v FucoPol, 25 mg of dry sterilized FucoPol were dissolved in 10 mL of test medium and left to agitate in an orbital shaker at room-temperature overnight.

A summary of all formulas prepared is presented in Table III.2. Beware of the fact that viability outcome depends on the salts used to produce the ionic composition. Therefore, as different preparation methods to achieve the same final composition may yield different results, preparation procedure is specified.

The DMEM stock solution is a complete culture media and was obtained from Sigma-Aldrich, consists of the core components of Dulbecco (+) as shown in Table III.2 and was supplemented with 1 g/L D-glucose and 1% w/v sodium pyruvate from Gibco, 3.7 g/L sodium bicarbonate from Sigma-Aldrich, 1% w/v GlutaMAX™ (L-alanyl-L-glutamine dipeptide) from Life Technologies and 100 U/mL penicillin, 100 µg/mL streptomycin and 10% fetal bovine serum (FBS) from Invitrogen. The DMEM (-) stock solution is identical to DMEM but does not contain 10% FBS.

Dulbecco (+), also known as D-PBS, is a nutrient-rich, PBS-like formulation that pioneered the complete DMEM solution now widely used[148]. Preparation involved adding 0.20 g $CaCl_2$, 0.20 g KCl, 0.20 g K_2HPO_4, 0.98 g $MgSO_4.7H_2O$, 8.0 g NaCl and 2.16 g $Na_2HPO_4.7H_2O$. Instead of adding KH_2PO_4, its conjugate base K_2HPO_4 was added. For more on this, refer to the footnote in Table III.2.

Dulbecco (-), also known as D-PBSA, is the Ca^{2+}-free, Mg^{2+}-free variant of D-PBS. Preparation was identical to its (+) counterpart, with the exception that $MgSO_4.7H_2O$ was not added. Instead of adding KH_2PO_4, its conjugate base K_2HPO_4 was added. For more on this, refer to the footnote in Table III.2.

Earle's Balanced Salt Solution is designed to have high bicarbonate content and withstand elevated CO_2 levels[148]. Preparation involved adding 0.02 g $CaCl_2$, 0.40 g KCl, 0.20 g $MgSO_4.7H_2O$, 6.68 g NaCl, 2.20 g $NaHCO_3$, 0.14 g $NaH_2PO_4.H_2O$ and 1.0 g D-glucose.

Hank's Balanced Salt Solution is the antagonist of Earle (content-wise), whereas a low bicarbonate concentration is present to avoid the need to incubate flasks in a CO_2 gas phase[148]. Preparation involved adding 0.14 g $CaCl_2$, 0.40 g KCl, 0.06 g K_2HPO_4, 0.10 g $MgCl_2.6H_2O$, 0.10 g $MgSO_4.7H_2O$, 8.0 g NaCl, 0.35 g $NaHCO_3$, 0.09 g $Na_2HPO_4.7H_2O$ and 1.0 g D-glucose. Instead of adding KH_2PO_4, its conjugate base K_2HPO_4 was added. For more on this, refer to the footnote in Table III.2.

Spinner-MEM is a variation of Eagle's MEM medium designed for cell suspension procedures and is purposely deficient on Ca^{2+} in order to reduce cell aggregation and adherence[148]. Preparation involved adding 0.40 g KCl, 0.20 g $MgSO_4.7H_2O$, 6.8 g NaCl, 2.20 g $NaHCO_3$, 1.40 g $NaH_2PO_4.H_2O$ and 1.0 g D-glucose.

Table III.2. Final composition of each normothermic formulation produced from scratch. All these solutions are originally extracellular-fluid type solutions (ECF), but were skewed slightly to an ICF-type character by increasing the amount of K^+. Shorter designations for each solution were adopted for convenience.

	Constituent *(mM)*	*Dulb (+)	*Dulb (-)	Earle	*Hank	S-MEM
IONIC	Na^+	153.0	153.0	141.5	141.7	152.7
	K^+	4.2	4.2	5.4	5.8	5.4
	Ca^{2+}	1.8	-	0.2	1.3	-
	Mg^{2+}	4.0	-	0.8	0.9	0.8
	Cl^-	143.2	139.6	120.0	145.9	121.8
BUFFER	$H_2PO_4^-$	1.5	1.5	1.0	0.4	10.1
	HPO_4^{2-}	8.1	8.1	-	0.3	-
	SO_4^{2-}	4.0	-	0.8	0.4	0.8
	HCO_3^-	-	-	26.2	4.2	26.2
SUGAR	Glucose	-	-	5.6	5.6	5.6

* Instead of adding KH_2PO_4, its conjugate base K_2HPO_4 was added, as literature mentioned that ICF-type solutions with greater K^+ content slightly increased viability[148]. More than a change in viability, a displacement of pH in equilibrium would most likely occur. However, pH was monitored and remained within physiologically acceptable values.

4.5. Animal cell lines

To assess the cryoprotective performance of FucoPol in different cell lines, the procedure indicated in Figure III.3 was employed to Vero, Saos-2, HFFF2 and C2C12 cell lines. A detailed description of each cell line can be found in Table III.3. Each cell line was exposed to DMEM alone or supplemented with 0.25% w/v FucoPol, viability was assessed and compared only within each cell line. To prepare DMEM + 0.25% w/v FucoPol, 25 mg of dry sterilized FucoPol were dissolved in 10 mL of DMEM and left to agitate in an orbital shaker at room-temperature overnight.

Table III.3. Different cell lines, specifications and culture medium used. Given their different growth rates, each cell line was assayed separately to avoid cross-contamination in the microplate and therefore provide accurate viability results for a single cell type.

Cell line	Growth medium	Description
Vero (ATCC® CCL-81™)	DMEM	Kidney epithelial cell line from an adult African green monkey (*Chlorocebus sabaeus*), initiated in 1962 at the Chiba University in Japan.
Saos-2 (ATCC® HTB-85™)	McCoy	Human osteoblast cell line isolated from the osteosarcoma of an 11-year old Caucasian girl by J. Fogh and G. Trempe.
HFFF2 (ECACC 86031405)	DMEM	Human fetal foreskin fibroblast cell line derived from a 14-18 week-old fetus. This cell line has a finite life span.
C2C12 (ATCC® CRL-1772™)	DMEM	Mouse myoblast cell line derived from H. Blau as a subclone of the original cell line established by D. Yaffe and O. Saxel.

5. Antioxidant properties

5.1. Reducing power assay: $K_3Fe(CN)_6$

For determination of the reducing power of FucoPol, an adaptation of the method pioneered by Oyaizu *et al.* (1986) was employed[149]. To a test tube, 250 µL of 200 mM PBS pH 6.6, 250 µL of 1% w/v $K_3Fe(CN)_6$ and 250 µL of sample were added. FucoPol was prepared in concentrations of 1, 0.8, 0.6, 0.4, 0.2, 0.1 and 0.05% w/v in distilled water. The mixture was briefly vortexed until homogenized and then left to incubate at 50ºC for 20 min. Then, 250 µL of 10% w/v trichloroacetic acid (TCA) were added. The mixture was centrifuged at 2375 *g* for 10 min and a biphasic system formed. To the collected upper layer, 50 µL of 0.1% w/v $FeCl_3$ were added. After 10 min of reaction time, absorbance at 700 nm was measured. Distilled water was used as a blank reference and ascorbic acid (vitamin C), of equal concentration range, as the positive control. For a mechanistic interpretation of the procedure, refer to Section III.8.5.

To prepare 200 mM PBS pH 6.6, 1.68 g of $NaH_2PO_4.H_2O$ and 2.09 g of $Na_2HPO_4.7H_2O$ were dissolved in 80 mL of distilled water. The pH was corrected with 1 M HCl from 6.8 to 6.6 and the remaining amount of distilled water was added for a final volume of 100 mL.

To prepare 10% w/v TCA, 10 g of TCA were dissolved in 100 mL of distilled water. Then, the volume was split by triplicates of 1.5 mL eppendorf tubes and quickly centrifuged. The supernatant was collected as the working solution for the experiment.

To prepare 0.1% w/v $FeCl_3$, 0.017 g of $FeCl_3$ were dissolved in 50 mL of distilled water. The solution was prepared right before its experimental use and must be stored in a dark environment.

To prepare 1% w/v ascorbic acid, 1 g of L-ascorbic acid was dissolved in 100 mL of distilled water. Then, successive 2-fold dilutions were performed to produce the working concentrations. All samples were stored at 4ºC in a dark environment.

5.2. Reducing power assay: Fe^{3+}-TPTZ

The ferric-reducing antioxidant potential (FRAP) of FucoPol was determined with an adaptation of the original experiment[150]. A working FRAP solution (WFRAP) was prepared by mixing 25 mL of 0.3 M acetate buffer, 2.5 mL of 0.01 M 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) and 2.5 mL of 0.02 M $FeCl_3.6H_2O$. After homogenization of the initial mix, a 270 µL aliquot was collected, to which 27 µL of distilled water and 9 µL of sample were added. The test mixture was quickly vortexed, incubated at 37ºC for 30 min and its absorbance at 595 nm was measured. As test samples, FucoPol

concentrations of 1, 0.8, 0.6, 0.4, 0.2, 0.1 and 0.05% w/v were prepared by successive 2-fold dilutions. Distilled water was used as a blank reference. Trolox, a water-soluble analog of vitamin E, of concentrations 300, 250, 200, 150, 100 and 50 µM, was used as positive control. For a mechanistic interpretation of the procedure, refer to Section III.8.6.

To prepare 0.3 M acetate buffer, 1.6 g sodium acetate were dissolved in 8 mL of glacial acetic acid. The solution was stored at 4ºC until use.

To prepare 0.01 M TPTZ, 312 mg TPTZ were dissolved in 100 mL of a previously prepared 0.04 M HCl solution. The solution was stored at 4ºC in a dark environment.

To prepare 0.02 M $FeCl_3.6H_2O$, 540 mg of $FeCl_3.6H_2O$ were dissolved in 100 mL of distilled water. The solution was prepared right before its experimental use and must be stored in a dark environment.

5.3. *In vitro* experiment: H_2O_2-mediated ROS induction

To assess the antioxidant potential of FucoPol in a living system, Vero cells were cultured as detailed in Figure III.3 and underwent a reactive-oxygen species induction assay at 37ºC[151]. After the cell seeding stage, Vero cells were pre-incubated with either DMEM or DMEM + 0.25% w/v FucoPol for 1 h. Then, the cells were exposed to 300 µM of H_2O_2 for 3, 6 and 24 h. Two negative control conditions (one for each medium) of non-exposure to H_2O_2 were performed as well.

The H_2O_2 additions were done in a countdown fashion – the first addition accounted for the 24-hour mark, then the 12-hour mark, and so on – so that the addition of the viability indicator would be done at t = 0. Then 10 µL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were added to each well. After 3 h of incubation, the liquid contents of each well were discarded and 100 µL of pure DMSO were added. Shortly after, absorbance at 570 and 690 nm was measured. For a mechanistic interpretation of the procedure, refer to Section III.8.7.

To prepare 300 µM H_2O_2, 67 µL of a 10 mM H_2O_2 stock solution were added to 933 µL of distilled water for a final 10 mL solution of 3.3 mM H_2O_2. Then, 10 µL of 3.3 mM H_2O_2 were added to 100 µL of medium in the well, resulting in a final H_2O_2 exposure concentration of 300 µM. Added exposure volumes of H_2O_2 must be small to not dilute the cell media, thus accurately reflecting their antioxidant potential.

To prepare 5 mg/mL MTT, 500 mg of MTT were dissolved in 100 mL of DMEM and sterilized with 0.2 µm attachable syringe filters to a new Falcon tube. The solution was stored at 4ºC in a dark environment.

6. Combinatorial substitution

6.1. Knock-out and gradient-based substitutions

In order to evaluate how the addition of FucoPol to a cryoprotective formulation could compensate the absence of other constituents, two simple experiments were designed. This section is a brief explanation on what insight a knock-out or a gradient-based substitution can give in practical terms. Some constituents are very expensive to implement, which increase the final value of the formula, or frowned upon by the biomedical community, such as animal-derived products. The

idea here is to balance viability with solution simplicity, and the goal is to achieve the highest viability possible with the lowest number of constituents. In both methods, the core ionic composition does not vary – that itself is assessed by using different formulations.

In knock-out substitutions, a target constituent of a cryoprotective formula is strategically removed. With its removal and posterior addition of 0.25% w/v FucoPol, one can infer if there is a compensation effect by evaluating post-thaw cell viability. Then, incremental eliminations are performed, removing more than one class of constituents but maintaining constant the amount of FucoPol added.

In gradient-based substitutions, only one constituent is targeted for removal. A gradual elimination of that substance, testing a range of 100 to 0% of its original quantity in the final composition is performed, while adding 0.25% w/v FucoPol at each elimination step. This method allows to determine a threshold concentration at which either (i) no further compensation by FucoPol is present or (ii) a negative outcome starts to be observable due to insufficient fraction of the constituent; or both.

For a list of all substitutions tested in this study, refer to Table IV.6, as it is a valuable interpretative asset of the results.

6.2. Hypothermic formula preparation

Contrary to the normothermic formulas studied in Section IV.4.3 – which are optimized to ensure successful cell growth at 37ºC – three hypothermic formulas, designed to protect cells from the hypothermic continuum, were prepared from scratch. Most hypothermic formulas are ICF-type solutions, characterized by a high K^+/Na^+ ratio and intended to prevent cellular edema by maintaining intracellular homeostasis of the Na^+/K^+ ATP-pumps upon cold-induced dysfunction[96].

These test formulas are simplified adaptations of the originally sold products, given the trade secret surrounding their composition, but provide nevertheless a basis for formula optimization studies. Table III.4 provides a comparison between the original and adapted compositions used in this study.

To produce the hypothermic solutions, dissolution was performed under continuous slow magnetic stirring and subsequent additions were only carried out after the solution had homogenized at each step. The pH was confirmed to be in the 6.8–7.2 interval and the solutions were stored at 4ºC for no more than 1 month until use.

To prepare 1 L of Euro-Collins in distilled water, the following reagents were added in order: 0.84 g $NaHCO_3$, 1.12 g KCl, 2.04 g KH_2PO_4, 7.4 g K_2HPO_4 and 34.95 g D-glucose.

To prepare 1 L of Unisol-CV in distilled water, the following reagents were added in order: 5.55 mg CaCl2, 3.05 g $MgCl_2.6H_2O$, 23.4 g potassium gluconate, 1.26 g $NaHCO_3$, 0.29 g NaCl, 3.45 g $NaH_2PO_4.H_2O$, 3.34 g $Na_2HPO_4.7H_2O$, 0.9 g D-glucose, 5.13 D-sucrose, 4.55 g D-mannitol and 60 g dextran-40 (MW = 40,000 Da).

To prepare 1 L of Custodiol-HTK in distilled water, the following reagents were added in order: 1.66 mg $CaCl_2$, 0.81 g $MgCl_2.6H_2O$, 0.88 g NaCl, 0.75 g KCl and 5.47 g D-mannitol.

Table III.4. Hypothermic formulas used as control media for the combinatorial substitution experiment and their deviations from published compositions, due to reagent constraints. Minor ion content deviations, arise from a mass balance that differs from the original work, as some information was possibly hidden. Therefore, the present working compositions were the best achievable in the laboratory, given the limited information available in literature. The supplements section is an extension of the original composition and were not added to the formulas, as most were long-term (months to years) preservation supplements.

Constituent		Euro-Collins		Unisol-CV		Custodiol-HTK	
		Published[152]	This work	Published[152]	This work	Published[89]	This work
IONIC	Na^+	10 mM		60 mM	70 mM	15 mM	
	K^+	115 mM		70 mM	100 mM	10 mM	
	Ca^{2+}	---		0.05 mM		0.015 mM	
	Mg^{2+}	---		15 mM		4 mM	
	Cl^-	15 mM		30.1 mM	35.1 mM	50 mM	33.03 mM
BUFFER	$H_2PO_4^-$	15 mM		---	25 mM	---	
	HPO_4^{2-}	42.5 mM		---	12.5 mM	---	
	HEPES	---		35 mM	---	---	
	HCO_3^-	10 mM		5 mM	15 mM	---	
IMPERMEANT	Glucose	194 mM		5 mM		---	
	Sucrose	---		15 mM		---	
	Mannitol	---		25 mM		30 mM	
	Gluconate$^-$	---		70 mM	100 mM	---	
	Lactobionate$^-$	---		30 mM	---	---	
COLLOID	Dextran-40	---		6% w/v		---	
SUPPLEMENTS				**Adenosine** *Energy substrate*		**Ketoglutarate** *Energy substrate*	
				Glutathione *Antioxidant*		**Histidine** *pH buffer*	
						Tryptophan *Membrane stabilizer/antioxidant*	

7. Preliminary tests

7.1. Minimal cryoprotective FucoPol concentration threshold

In order to minimize the amount of FucoPol needed to observe significant cryoprotection, FucoPol solutions of 0.25, 0.13, 0.06 and 0.03% w/v were prepared in DMEM. Then, Vero cells were seeded in a 96-well microplate as detailed in Figure III.3 and exposed to the different media. DMEM without FucoPol was used as the negative control medium.

7.2. Cryoprotection outcome dependency on fucose composition

To assess how the composition of a polysaccharide contributes to the cryopreservation phenomenon, four alternative biopolymers produced by colleagues were assayed. In order to sterilize each polymer for biological use, the standardized procedure for FucoPol was implemented and is detailed in Section III.4.1. By order of decreasing fucose content, 0.25% w/v of FucoPol, A, B, C and D polymers were diluted in DMEM and exposed to Vero cells, for a total of 5 compositions tested. The assay was carried out as detailed in Figure III.3 and DMEM without any polymer was used as the negative control medium.

7.3. Assessing the CTN effect of FucoPol on DMSO

To understand if FucoPol could have a cryoprotective toxicity neutralization effect on DMSO, an assay identical to what is detailed in Figure III.3 was performed, but only at 37ºC to potentiate its toxicity mechanism. Vero cells were seeded in DMEM and exposed to 0, 10 and 20% v/v DMSO in the absence or presence of 0.25% w/v FucoPol. Instead of the 30 min incubation time at 37ºC, 5% CO_2, exposure times were extended to 1, 3 and 5 h. The exposure to DMSO was prepared in a countdown fashion, just like what is detailed in Section III.5.3. DMEM without FucoPol or DMSO was used as the control medium.

7.4. Implementation of FucoPol in CryoStor™

To evaluate if FucoPol could enhance the performance of a marketed formulation, CryoStor™ CS5 was obtained from BioLife Solutions, LOT #18054. Both the original CryoStor™ solution (CS) and one supplemented with 0.25% w/v FucoPol (CS+FP) were used as media for Vero cells, which were subject to the cryo-assay detailed in Figure III.3. DMEM was used as the control medium in the presence and absence of 0.25% w/v FucoPol, but was instead supplemented with 5% v/v DMSO instead of the regular 10%, to mimic the DMSO content of CryoStor™ CS5.

7.5. Photoabsorptive properties of FucoPol

To assess the absorptive properties of FucoPol to near-UV and visible radiation due to its opaqueness, a UV-VIS spectrum spanning the 250–750 nm range of a 0.25% w/v FucoPol was recorded in a VWR V-1200 spectrophotometer using a Hellma Analytics 10 mm quartz cell.

Then, an *in vitro* experiment was designed to determine if FucoPol could prevent incident UV radiation of damaging a cell layer. Vero cells underwent an identical pre-treatment stage as detailed in Figure III.3, with two exceptions: (i) cells were cultured in four 10 mL Petri dishes for a final concentration of 100,000 cells/cm^2 and (ii) an additional 24 h incubation step at 37ºC, 5% CO_2 was performed to promote cell adhesion.

After seeding, four conditions were tested. Vero cells were either immersed in DMEM or DMEM + 0.25% w/v FucoPol and irradiated for 30 min in a laminar flow chamber with a 254 nm ultraviolet lamp. The non-irradiated controls were left to incubate at 37ºC, 5% CO_2 for the same amount of time. After the procedure, viability assessment was performed with resazurin after the regular 2 h and an additional 24 h incubation period, upon which absorbances were collected. A representation of the experimental set-up is alternatively presented in Section IV.8.5 as it provides a visual aid for discussion.

8. Auxiliary assay interpretation

8.1. Following growth of bacterial cells by A_{450}

The quantification of bacterial cells in the bioreactor broth was followed by measuring the absorbance at 450 nm. The chosen wavelength is largely dependent on the turbidity of the solution and ranges from 420 – 660 nm[153]. From the growth of *Enterobacter* A47, the maximum absorbance value can be found at 450 nm, which explains its use. If A_{450} > 0.3, the sample is diluted until absorbance is between valid criteria to avoid skewed results.

8.2. Hemocytometer cell counting rules

During cell count after trypan blue staining, there are specific rules for accurately extrapolating the correct amount of cells cultured from a partial subset in the Neubauer chamber, or hemocytometer. A hemocytometer chamber contains 5 counting squares, one in the middle surrounded by the remaining four. The counting system is arbitrary, as long as it is respected for every counting experiment, but some conditions[154] are universal:

1. Inside a square, every individual cell is counted. Cells are usually spherically and present a blue coloring due to trypan blue.

2. For cells that are standing on the edge of a square, only two sides are included while the others are not counted. For example, cells on the north and west edges are counted while any cells on the south and east edges of the square are discarded, to avoid overextrapolation.

3. Sometimes, trypsinization does not individualize every single cells and aggregates may still be present. For this experiment, any aggregate that contains up to 3 cells was counted (as 3 individual cells) whilst aggregates with 4 or more cells were discarded.

4. Smears, dust particles or fibers can be present are present similarities to a cell. While this is rare and does not influence the final count, only fully spherical cells were classified as biological material, while any other non-spherical shape was discarded.

5. The final count was the average of 10 counting squares, from both chambers of the hemocytometer.

For a graphically simplified representation of the hemocytometer, refer to Figure III.3, where some cells are not counted (marked with an X), as stated by the rules above.

8.3. Resazurin cell viability assessment

Resazurin, commercially sold as alamarBlue®, is a blue dye above pH 6.5 that, in the presence of reducing agents such as the mitochondrial pair exchanger NAD(P)H/NAD(P)$^+$, is irreversibly reduced to resorufin, of pink coloring[155]. Viability assessment using resazurin is usually performed by fluorescence measurement and results can be obtained in 1 h. However, absorbance at 570 (for resorufin) and 600 nm (for resazurin) can also be determined as resazurin has one of the highest values on the Kreft dichromaticity index[156], which means its hue change from blue to pink is easily perceived with the naked eye. Resazurin reduction is proportional to aerobic respiration, providing a good indication of metabolic viability of cells.

8.4. MTT cell viability assessment

MTT is an equally efficient viability indicator, but relies on reagent precipitation and resuspension. In the presence of NAD(P)H-dependent cytosolic oxidoreductase enzymes, MTT is reduced to the insoluble formazan, which has a purple color[157]. Solubilization is then done with DMSO or acidic reagents, yield a purple colored solution that can be quantified by measuring the absorbance at 570 nm (and 690 nm for background subtraction). Quantification by MTT depends on the metabolic rate of cells because it results from a NAD(P)H flux of reducing agents.

8.5. Oxidative mechanism of $Fe(CN)_6^{3-}$

Potassium ferricyanide is a source of oxidized iron that will be reduced in the presence of an antioxidant sample, therefore being valuable for determining the antioxidant potential of a sample. In general, an antioxidant is a reducing agent capable of scavenging oxidized species capable of self-reduction by oxidation of biological structures, like membrane lipids or proteins. After the Fe^{3+} to Fe^{2+} redox reaction, a second source of Fe^{3+} is added in the form of $FeCl_3$ to produce a Fe(II)-Fe(III) coordination complex that maximally absorbs at 700 nm. Complex formation is proportional to the amount of initial Fe^{3+} that was reduced by the sample, thus it represents its reducing power[158].

8.6. Oxidative mechanism of Fe^{3+}-TPTZ

The TPTZ reagent provides another validation tool to observe ferric reducing antioxidant power. The ferric form of the reagent, Fe^{3+}-TPTZ is reduced to the ferrous form Fe^{2+}-TPTZ in the presence of an antioxidant sample, a reaction macroscopically observable by the development of an intense blue color. The reaction is carried out in acidic environments and is non-specific, which means the reducing power reflects all electron donating species that have a higher redox potential than the Fe(III)-Fe(II) conversion[158]. Nevertheless, quantification is done at 595 nm due to maximal absorption and does not require the formation of a coordination complex like the previous assay.

8.7. Oxidative mechanism of H_2O_2-mediated ROS induction

Hydrogen peroxide might be one of the most central products of cell metabolism in the mytochondria, therefore it is useful to mimic the oxidative damage a cell suffers post-thawing without necessarily enacting the whole cryopreservation procedure. In a scenario of metabolic overload[159], NAD(P)H is oxidized to $NAD(P)^+$ at higher rates in complex I of the mitochondrial respiratory cascade, which in the presence of oxygen leads to $O_2^{\cdot-}$ radical formation. Cells have enzymatic antioxidant mechanisms for such events, and the SOD2 (superoxide dismutase) protein converts this radical into H_2O_2, the latter being neutralized to H_2O by glutathione reductase-derived enzymes such as peroxiredoxins and GPX1. However, activation of these enzymes is also NAD(P)H dependent, which quickly depletes in complex I. Gradually, the amount of ROS produced surpasses the enzymatic antioxidant resources of the cell and damage will occur due to accumulation. Free H_2O_2 will result in mitochondrial DNA damage, protein oxidation and membrane lipid peroxidation. Excess H_2O_2 can also diffuse through aquaporins and populate the cytosol, where it acts in thiol oxidation-derived cellular signaling. However, excess H_2O_2 will result in nefarious effects of uncontrolled differentiation and proliferation, as well as biased metabolic adaptation. H_2O_2 can also react with Fe^{2+} or Cu^+ to produce $OH^{\cdot}$.

9. Statistical analysis

All chemical and biological quantitative experiments are reflected by at least 3 or 5 replicate values and the results were expressed as mean ± standard deviation (SD). Error bars in graphs show ±1 SD and were produced in Microsoft Excel and Tableau 10.5. Outlier values represented by an asterisk (*) were removed from the mean value but still account for the SD of each data point. When relevant, the significance of any differences between pairs of data sets was evaluated by Student's *t*-test in gretl software to aid in data interpretation. A p-value < 0.05 was considered to be statistically significant.

IV. RESULTS AND DISCUSSION

1. Obtaining FucoPol

The strain *Enterobacter* A47 is capable of synthesizing and excreting a fucose-rich exopolysaccharide (EPS) that has seen its potential recognized for several applications. The biopolymer is patented under the International Patent WO2011073874A4, along with its optimized production procedure. Its biomedical properties remained relatively unexplored, but some characteristics of FucoPol were recognized to be possibly useful in cryopreservation fluids, namely its shear-thinning behavior, thermal stability and biodegradability.

Then, exploring the potential implementation of FucoPol in the challenging field of cryobiology was a tempting objective, given that solutions for consistently securing biological viability are still lacking. Obtaining FucoPol was then the first step necessary to carry out an exploratory work on its applicability.

1.1. Bioreactor production

Bioreactor assays are automated experiments that rely on computational control of the bacterial growth parameters (pH, temperature, stirring speed and O_2 pressure) to maximize polymeric production. The method was optimized by Torres *et al*. in 2011 using glycerol as the carbon source and using continuous feeding as the cultivation mode[160].

Enterobacter A47 was reactivated in Chromagar plates. Figure IV.1 demonstrates the initial growth on solid medium, with a significant blue tonality representing the bacteria. The left plate presented the highest number of individual colonies and was used to obtain a superficial slice of the *Enterobacter* A47 colony. Then, the bacteria were transferred to liquid LB medium to obtain the pre-inoculum, on which they grew to a considerable cell density before being transferred to the Medium E* inoculum and, afterwards, to the bioreactor.

The solid-to-liquid transfer step is important for two reasons. The first is to guarantee that no contaminating species are present further ahead in the bioreactor. If two different strains are competing for the nutrient source, FucoPol production is not maximized and the purification steps become more complex. For the same purpose, the solid medium was observed after one week, truly confirming that the isolation of a single strain was successful. The second reason is to eliminate genetic drift: if all bacterial growth is derived from an individual colony instead of multiple, we can guarantee that all cells in the bioreactor will be descendants of a single parent cell, therefore normalizing how bacteria behave to yield the final result.

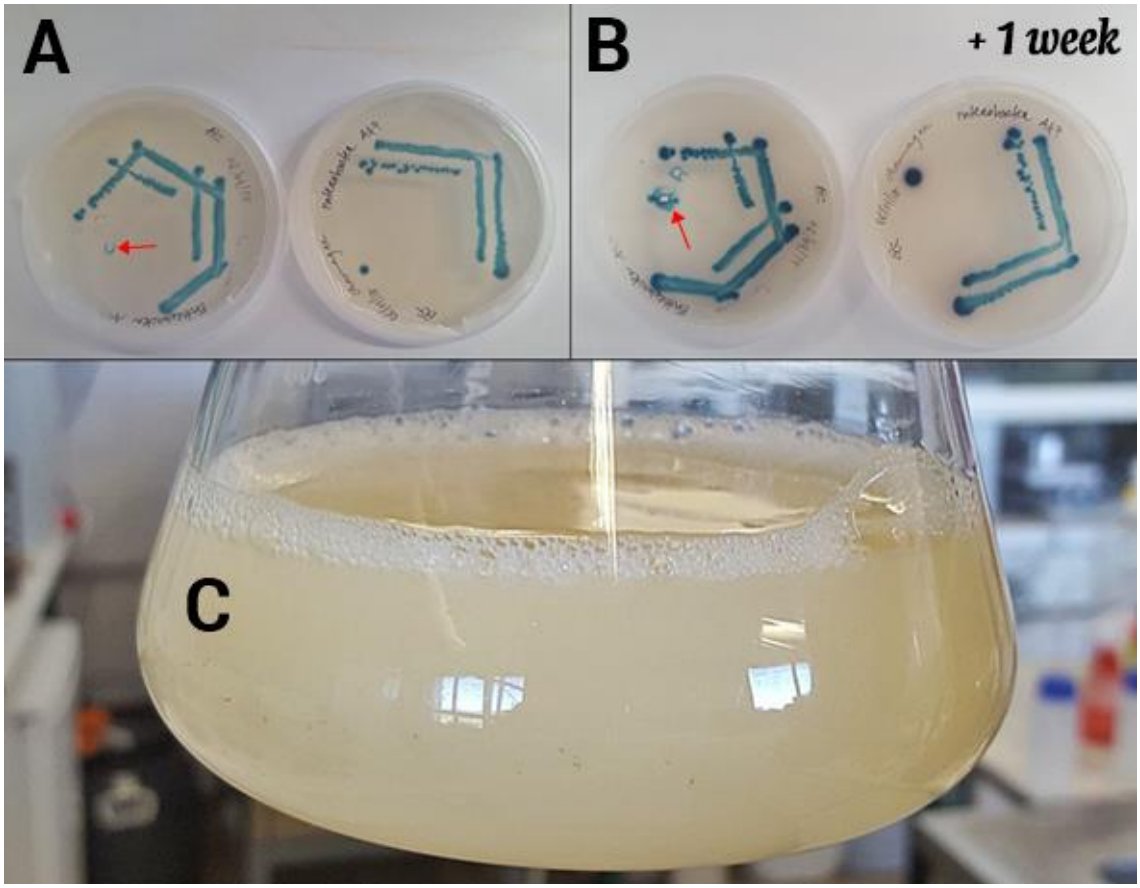


Figure IV.1. Selection, isolation and initial growth of *Enterobacter* A47. Bacterial colonies after 1 (A) and 7 days (B) of growth appear as blue due to the composition of Chromagar. The duplicate plates were observed after a week as quality control assurance of no cross-contamination. After transfer of an isolated colony to the LB medium, a beige color appears (C) in the pre-inoculum, characteristic of due to bacterial growth.

Bacteria are grown in medium E before bioreactor initiation, so bacteria can adapt to the nutrient source, which essentially is the same in the bioreactor chamber. After inoculum injection, the bioreactor procedure was monitored for *ca*. 4 days and the data collected is presented in Table IV.1.

The first day of production went accordingly to what is expected for bacterial growth. *Enterobacter* A47 did not present a latent phase of growth as it reacted prompty to nutrient availability. This is in accordance with the turbid beige tonality of the inoculum shake flask just before bioreactor inoculation. From the moment of inoculation to t = 8 h, a decrease in dO_2 was the only observable change in the broth, with a steady increase of A_{450}. This indicates that bacterial growth was occurring and consistently high amounts of oxygen were being consumed. However, apparent viscosity remained unchanged, which means at that point, no significant production of FucoPol was occurring. Throughout the experiment, the cultivation conditions (30ºC, pH 7, 10% dO_2) were automatically controlled. If any deviations from the set-points occurred, the device would compensate by different mechanisms: if pH changed, NaOH was added in accordance. Temperature was controlled by a thermostat and the stirring speed would vary in relation to dO_2.

Table IV.1. Growth parameters obtained from 0 to 75 h of the bioreactor run. Grey columns represent the production parameters periodically monitored. The blue column represents the assessment of bacterial growth by absorbance measurement at 450 nm. Orange columns represent the amount of FucoPol produced and the respective viscosity of the solution at the moment of collection. Columns with the Σ designation indicate that the values presented are cumulative over time. Cells with n.d. placeholders represented values that were not determined because the viscosity was outside the spindle's range. Viscosity values are approximate: for determination, refer to Section III.1.6.

Time *(h)*	Temp. *(°C)*	pH	ΣBase *(mL)*	dO_2 *(%)*	Stirring *(rpm)*	ΣAntifoam *(mL)*	ΣFeed *(mL)*	ΣA_{450} *(U/L)*	ΣFucoPol *(g/L)*	Viscosity *(mPa·s)*	Glycerol *(g/L)*	Ammonia *(g/L)*
0	30.0	6.96	9	79.2	308	0	0	0.42	0.12	1.34	37.6 ± 0.2	0.42 ± 0.03
1	30.0	6.98	10	73.8	308	0	0	0.47	---	---	---	
2	30.0	6.98	11	58.8	308	0	0	0.53	---	---	---	
3	30.0	6.98	13	32.6	308	1	0	0.59	---	---	---	
4	30.0	6.98	14	12	309	1	0	0.74	0.28	1.35	36.5 ± 0.6	0.35 ± 0.01
5	30.0	6.98	15	9.9	425	1	0	0.96	---	---	---	
6	30.0	6.98	16	9.8	497	1	0	1.48	---	---	---	
7	30.0	6.98	18	9.8	551	1	0	1.73	---	---	---	
8	30.1	6.97	23	10.2	649	1	0	2.53	0.27	1.38	32.7 ± 0.2	0.16
24	30.0	6.97	117	7.9	823	8	74	9.49	2.88	430.3	4.6 ± 0.1	0
27	30.0	6.98	128	9.9	788	8	88	9.68	4.36	*11266*	4.1 ± 0.1	0
30	30.0	6.97	138	10.2	733	8	100	10.53	5.28	*12713*	5.3	0
48	30.0	6.98	191	0.5	469	8	181	7.70	5.76	**n.d.**	3.6 ± 0.8	0
51	30.0	6.98	196	41.3	309	8	195	8.38	5.82	**n.d.**	3.9 ± 1.3	0
54	30.0	6.98	201	14.3	309	8	208	7.80	5.76	**n.d.**	3.4 ± 0.2	0
72	30.0	6.98	228	9.5	480	9	289	8.68	6.16	**n.d.**	3.4	0
75	30.0	6.98	231	4.2	526	9	303	9.91	6.56	**n.d.**	3.4	0

In day 2, an effort to maintain dO_2 above 10% resulted in the increase of the stirring speed. This is most likely due to the depletion of NH_4^+ in combination with an unbalanced intake of glycerol that resulted in a metabolic shift to carbon accumulation. It is safe to assume that FucoPol excretion to the extracellular medium started between 8 and 24 hours of cultivation. FucoPol production increased steadily to a maximum accumulation of 6.56 g/L at day 4. In fact, viscosity was so high that values measured became unreliable to the sensitivity of the viscosimeter.

The decrease in absorbance is probably correlated with the loss of broth volume that occurred overnight due to foaming overflow: the glycerol feed eventually diluted the broth and because bacterial growth was already halted at that point, A_{450} appears to decrease. However, due to the increase in viscosity which became technically impossible to assess from t = 48 h onwards, all other interconnected parameters became inconclusive. It is then difficult to ascertain if a dilution effect is what caused the apparent decrease of cell density.

Nevertheless, the bioreactor experiment was stopped after 75 h and the remaining 1.2 L of broth were collected for purification of the product.

1.2. Evaluation of growth conditions

To assess the efficiency of FucoPol production, the broth samples collected at each time point of Table IV.1 were used for the characterization of glycerol and ammonia intake by *Enterobacter* A47 and the results are shown in Figure IV.2.

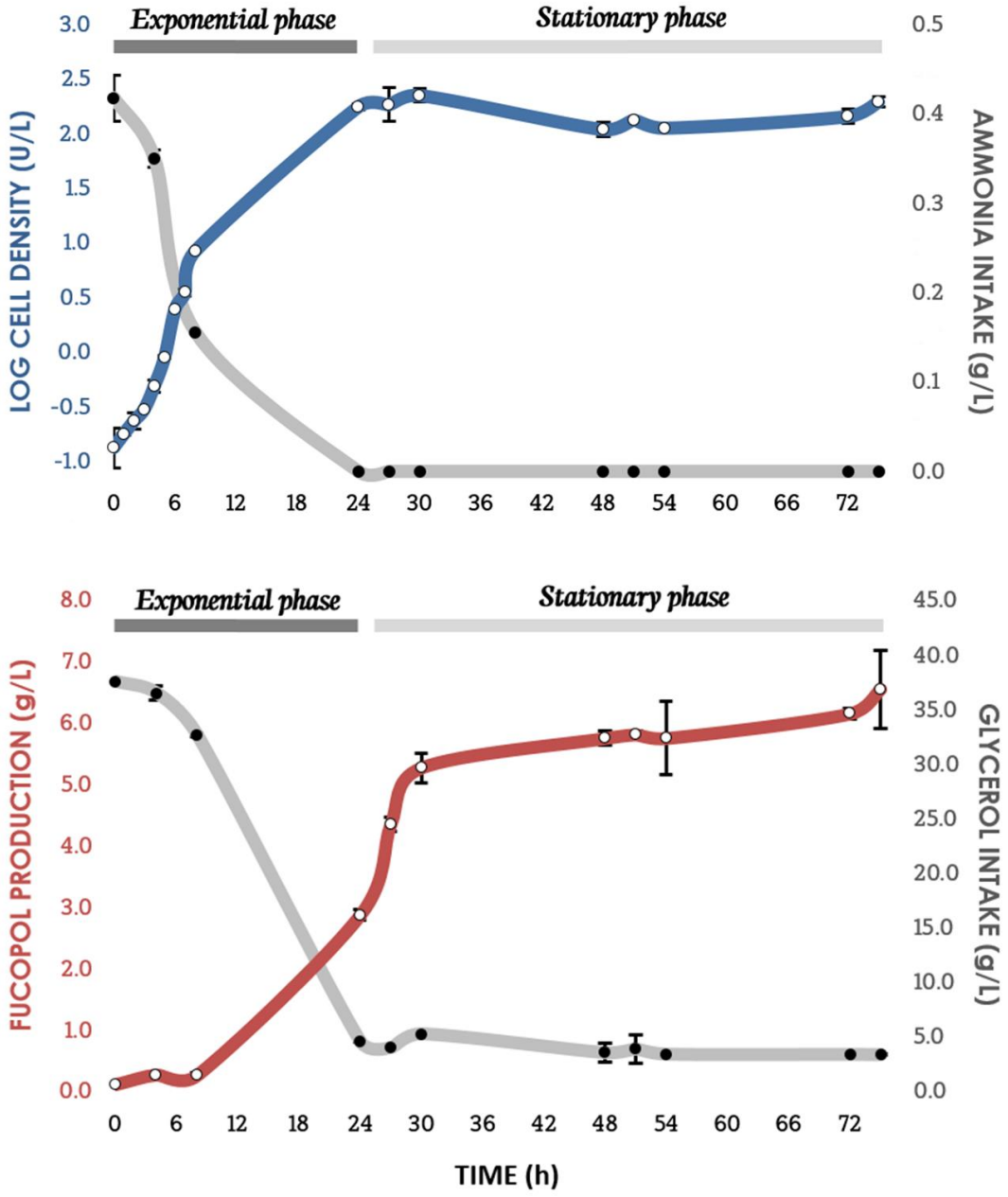


Figure IV.2. Contrasting evolution of *Enterobacter* A47 growth and FucoPol production over time. In blue, cell growth is correlated with nitrogen concentration (gray, calibration fit R^2 = 0.9963). In red, FucoPol production is correlated with the carbon concentration (gray, calibration fit R^2 = 0.9998). Notice the inflection in cell growth is then followed by an inflection in polymer excretion until both reach equilibrium.

Given that the growth profile of *Enterobacter* A47 lacks a latent phase, t = 0 h immediately corresponds to a developing exponential phase. Right after bioreactor initiation, ammonia concentration decreases in proportion to cell density because nitrogen is a resource for bacterial growth. Glycerol was only supplemented after 8 h but is steadily assimilated by *Enterobacter* A47 as well. As soon as ammonia was fully depleted, carbon abruptly decreased, which is consistent with the start of FucoPol production.

After the 30-hour mark and until the end of the procedure, both ammonia and glycerol were present in residual amounts. Notice that whilst nitrogen becomes fully depleted, which is the limiting factor for stopping cell growth, a residual 5 g/L of glycerol is still in the system. Considering that a starting concentration of 40 g/L glycerol were quickly assimilated just after a day, FucoPol production have been limited by an insufficient amount of carbon.

The continuous feeding mode employed is a strategical manipulation of the behavior of *Enterobacter* A47. This method relies on continuously feeding the broth with glycerol at a rate of 3.75 $g \cdot L^{-1} \cdot h^{-1}$ in a nitrogen-limiting environment. Because both nutrients are necessary for bacterial growth but only carbon becomes implemented in the exopolysaccharide produced, nitrogen-limiting conditions will induce *Enterobacter* A47 to shift its metabolic load from cell division to carbon accumulation in the form of FucoPol[161]. Therefore, by limiting nitrogen, it is possible to maximize polymer production while maintaining cell density relatively constant.

Defining the optimal parameters for FucoPol production envisioning maximum attainable content has been the focus of our group since 2008. This bioreactor yielded 6.56 ± 0.64 g/L of FucoPol and had a volumetric productivity of 2.10 g/L per day of experiment, which is comparable to previous studies[162,163,164]. In the best-case scenario, this means that in 75 h, *ca.* 6.6 g of FucoPol could have been produced, which is congruent with the amount of polymer dialyzed. An average net yield of 0.17 ± 0.02 $g_{EPS}/g_{Glycerol}$ was obtained which is in congruence to reported values of 0.10–0.17 at similar conditions[164].

The net yield shows the rate of conversion of the carbon source (glycerol, in this case) to FucoPol and provides an estimation of the metabolic efficiency and resource allocation of *Enterobacter* A47 in the tested conditions. A lower cell density could result in less FucoPol being produced, as values can reach up to 8.77 g/L in 3 days[139].

1.3. From broth to final product

After collecting 1.2 L of broth from the bioreactor, polymer extraction was performed by dia/ultrafiltration using a 100 kDa membrane. Dia/ultrafiltration involved several pre-treatment steps of the broth, including thermal denaturation of associated proteins and centrifugation of cellular biomass that was discarded.

Figure IV.3 shows the sequential evolution of the samples, from the opaque broth to a translucid treated cell-free supernatant. The first centrifugation step envisioned the removal of the bulk biomass from the broth, which was first diluted with deionized water for viscosity reduction. Then, a 70ºC incubation was performed for 1 h, to denature any residual proteins. To eliminate any possible contribution of polysaccharide-adhered proteins to further experimental results, this step was essential. A second centrifugation step was done to remove any residual biomass and denatured proteins. Then, lyophilization was performed and the cotton-like biopolymer was collected and stored at room-temperature in a sealed recipient.

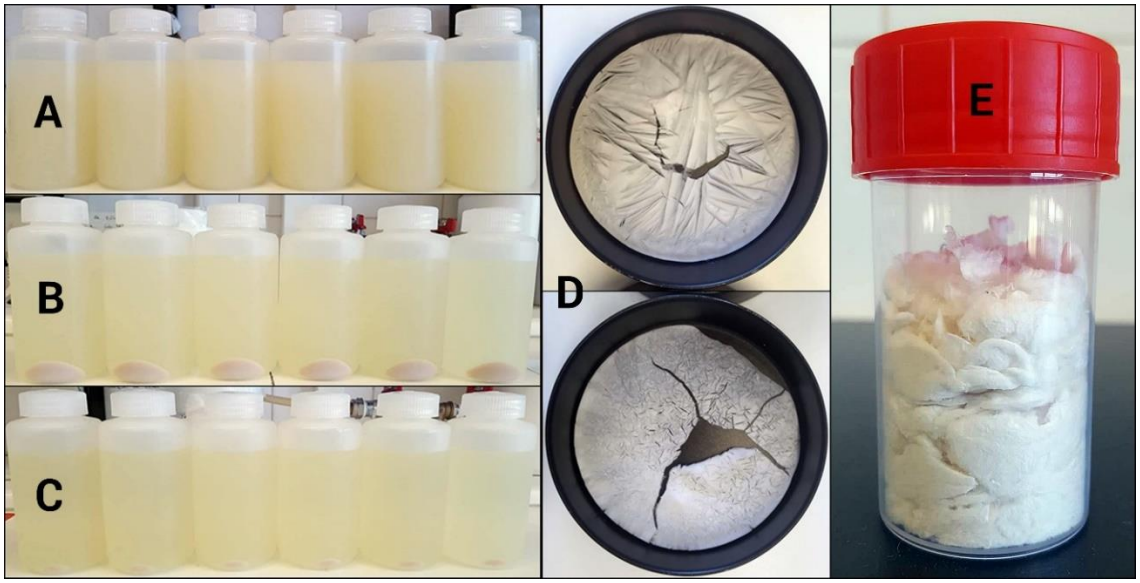


Figure IV.3. Visual evolution of the bioreactor broth along the purification steps. The initial diluted broth (A) was centrifuged to remove the cell pellet (B) and incubated at 70ºC to denature residual proteins (C). The supernatant was then lyophilized in two batches (D) and collected in a sample tube (E) for usage in all subsequent experiments. FucoPol has a cotton-like appearance.

Dialysis yielded an average 6.56 ± 0.64 g/L of FucoPol when compared to 4.32 g/L obtained from dia/ultrafiltration. Dia/ultrafiltration is based on a pressurized separation of the constituents from the broth in order to purify FucoPol, which might explain the polymer loss. The 100 kDa cut-off of the dia/ultrafiltration membrane ensures that FucoPol does not permeate and flows through the retentate back to the broth recipient (see Figure III.2). However, applied pressure to the pipelines induces shear stress on the polymer that may forcefully allow it to pass through the membrane and be lost to the sewer exit.

Although production was not maximal, 5.18 g of FucoPol were collected for further experiments, which was the main objective of this part of the study. Further on, exploratory research on the properties of FucoPol was employed to assess if it could meet the essential criteria for an efficient cryoprotection fluid.

2. Physicochemical properties

From this moment onward, FucoPol shall be graphically addressed by the symbol ✣ whenever substituting text by symbology is considered a visual improvement for simplifying the interpretation of tables and figures.

The main criterion in developing a cryoprotective formula based on FucoPol is that the polymer must not be conflictuous with the original solution, and an ideal scenario is if performance is enhanced. In assessing the adequacy of FucoPol to a formula, there are two types of interactions that must be evaluated: primary and secondary interactions.

Primary interactions define the behavior of other substances in the presence of FucoPol, that is, how the cryoprotective formula performs in the presence of the polymer. Secondary interactions are determined by the environment in which the FucoPol-loaded formula will be used. For example, hypothermic formulas are perfused into the vascular tissue which contains a diverse number of proteins and other important structures that could be negatively affected by the polymer.

An ideal scenario at first is if the polymer does not establish any harmful primary interactions: since the original formula is already biocompatible, chances are that the addition of the polymer might enhance its performance without being harmful to the environment, and therefore no negative secondary interactions exist as well.

2.1. FucoPol is stable in physiological conditions

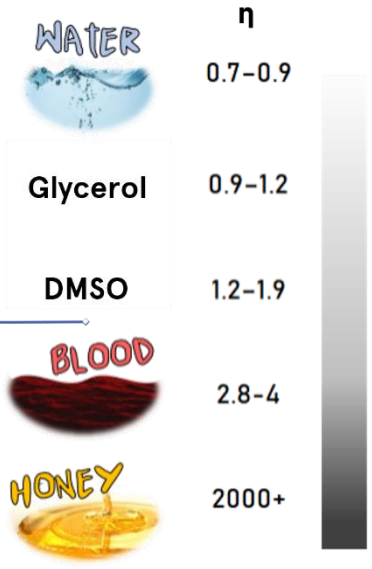
The simplest way to examine primary interactions is to supplement a cryoprotective formula with FucoPol and compare their properties. One of the least complex cryoprotective solutions, Euro-Collins was used as the basis for comparison (see composition in Table III.4). This formula is very simple and can be quickly produced with common laboratory reagents, as it only contains an ionic core composition, a pH buffer and glucose serving an impermeant purpose, that is, it acts as an osmolality regulator.

Secondary interactions are usually more difficult to assess because they require *in vitro* and even *in vivo* perfusion of the test formula to assess their practical success. However, we can indirect these interactions with albumin, as it is the most abundant protein in biological systems and constitutes 50% of the total blood proteome, acting as an oncotic regulator[165]. Because a FucoPol-based formula would be in contact with blood and interstitial fluids, bovine serum albumin (BSA) was used as a reference and an equal comparison was performed.

Table IV.2 presents both macroscopic and microscopic properties of all conditions tested. At first, FucoPol was simply dissolved in water to isolate its individual traits. Then, it was mixed with Euro-Collins or BSA in an attempt to evaluate primary and secondary interactions, respectively.

Table IV.2. Resulting macroscopic and microscopic traits of protein-polysaccharide and cryoformula-polysaccharide mixtures. To the right, a gradient of dynamic viscosities for several substances is shown for comparison. Notice that the Euro-Collins solution supplemented with FucoPol has a viscosity that is lower than blood. The viscosity interval corresponds to 37ºC and 25ºC, in ascending order. DMSO and glycerol values refer to 10% w/v, the standard concentration used in cryopreservation protocols. Viscosity values were obtained at room-temperature with an identical shear rate.

		+ Euro-Collins		+ BSA
MICROSCOPIC	pH	7.1	6.1	6.1
	η (mPa·s)	1.32	1.46	0.84
	Ω (µS/cm)	20	442	7830
MACROSCOPIC	Color	Milky beige	Milky beige	Milky beige
	Precipitate	No	No	No
	Homogeneity	Yes	-	Yes



Regarding the macroscopic features, either solution presented similar characteristics. The milky beige color was characteristic of the presence of FucoPol and was not an influencer of

performance. Two other characteristics were assessed: the presence of any precipitates and the degree of homogeneity. Despite being somewhat a cause and a consequence of each other, precipitation and homogeneity were arbitrarily classified as slightly different aspects.

Contrary to the absence of precipitates in solution which is quite a literal property to assess, the criteria for assessing homogeneity is based on the idea that the addition of FucoPol to either Euro-Collins or BSA would not disturb the solution's equilibrium state. Whilst precipitation can form solid pellets, a solution can still be heterogeneous if the contents are not fully mixed and presents fluctuating smears.

Neither solution formed any precipitates, but homogeneity was regarded as an absence of conflictive interactions. FucoPol does not present a classification for homogeneity as it is a single substance, but Euro-Collins and BSA in its presence yielded a homogeneous solution. It was assumed that negative primary and secondary interactions were not present and these constituents were compatible with FucoPol, but validation should be done *in vitro* or *in vivo* as macroscopic observation of a physical property is still insufficient at this point.

2.2. Viscosity is adequate for perfusion formulas

In what concerns microscopic properties, pH, dynamic viscosity and conductivity were assessed. In a qualitative approach, pH and the apparent viscosity were considered the most important parameters and overall, all solutions were quite similar. Conductivity might be a defining factor in membrane permeability: given the example of an organ, successful cryopreservation implies that the cryoprotective formula must reach inner cells by the interstitial spaces. This transmigration is dependent on conductivity[166] but conclusive interpretations can only be taken from cell assays. Based on previous knowledge presented in Chapter II, an increased ionic strength may not structurally affect FucoPol, but information on structure is usually derived from a functional parameter of FucoPol, which is viscosity. Nevertheless, conductivity results of each solution are still shown.

FucoPol in water or in the presence of BSA shows similar pH values of 6.1. While physiological conditions are usually between 6.8 and 7.2, cryoprotective fluids have pH buffers that counteract acidosis so this value does not reflect an *in vivo* scenario. It is interesting to see that FucoPol-loaded Euro-Collins is within that range (7.1), just as the original composition (data not shown).

Apparent viscosity is the most interesting parameter assessed, because perfusion is highly dependent of the solution's ability to flow freely through the vascular system. A turbulent flow could cause mechanical damage or even rupture of more sensitive blood vessels like capillaries, but a highly-viscous laminar flow would result in vascular clogging, causing ischemia. FucoPol alone had an apparent viscosity of 1.46 mPa·s and this value surprisingly decreased when the polymer was added to either Euro-Collins (1.32 mPa·s) or BSA (0.84 mPa·s).

In the polymer-protein mixture, a decrease to 0.84 mPa·s was observed. Jachimska *et al.* reported a decrease of the hydrodynamic radius of BSA above pH 4, which is consistent with a decrease of viscosity as a measure of resistance to flow[167]. Protein-protein or polymer-polymer crosslinking, which also provides attrition to flow, could have been eliminated by this heterogeneous interaction, reducing the viscosity. The observed measurement can then be a consequence of both a decrease in the hydrodynamic radius of FucoPol and/or BSA by some interaction which lacks elucidation at this point.

The polymer-formula mixture presented the most promising result. A small decrease in viscosity is actually an ideal scenario: the change in the formula's viscosity is not extreme but is sufficient to consider the addition of a polysaccharide to tailor its rheology for different applications. For example, different organs are subjected to different blood pressures, thus using a standardized cryoprotective formula for both a heart and a venal graft might not be advised. This ability to tailor the viscosity of a solution with small amounts of FucoPol avoids having to design a new formula, as it is done very often just to comply to one variable. From the viscosity gradient presented in Table IV.2, it is noticeable that the FucoPol-loaded Euro-Collins solution had a viscosity similar to 10% DMSO, which is widely used as the gold standard for cryopreservation. Given that blood viscosity at 37ºC is 2.8 mPa·s, perfusing this cryoprotective formula in blood vessels with an adjusted flow could greatly reduce any mechanical injury.

Whether a FucoPol-based formula is used as an immersion or perfusion fluid, some advantages of its use can already be extrapolated. The first one relates to reduced viscosity. Viscosity is a temperature-dependent property: for most fluids, the lower the temperature, the higher the viscosity simply due to mobility hindrance. However, because FucoPol has a shear-thinning behavior, an increase in shear stress at lower temperatures actually results in a lower apparent viscosity than expected from a linear relationship. If this is also the case for subzero temperatures, which is still unknown, then hypothermic perfusion could be even more efficient than common formulas because viscosity would not be as high. Another advantage is nutrient homogeneity. In Subsection I.6.3.1 it was mentioned that two of the major issues in cell to organ scale-up is temperature and nutrient heterogeneity. The ability of the formula to easily flow through the vasculature would facilitate nutrient suppliance to cells.

3. Antifreeze properties

Given that the main focus of this thesis was to implement FucoPol in a cryoprotection fluid formula, the most important property of the polymer was to have antifreeze activity. Viscosity is directly involved in hampering crystal growth and is the fundamental basis for vitrification. Given the high molecular weight of the polymer and its high apparent viscosity only at 1% w/v, it was postulated that it could prevent ice formation to a certain extent.

3.1. Flash freezing assay

The first proof-of-concept was a simple experimental design known as the flash freezing assay[168]. The concept is similar to the production of protein crystals for X-ray crystallography: the protein solution is mixed with glycerol at a given concentration so that glycerol prevents the growth of water crystals and a single protein crystal is obtained.

In this experiment, FucoPol solutions with concentrations ranging from 0.06 to 1% w/v were placed in the tip of a pipette. Deionized water and 50% v/v glycerol were used as the negative and the positive controls, respectively. The samples were then immersed in liquid nitrogen (-196ºC) and submersed almost subsequently. An experimental design coupled with photographic evidence of the results is shown in Figure IV.4.

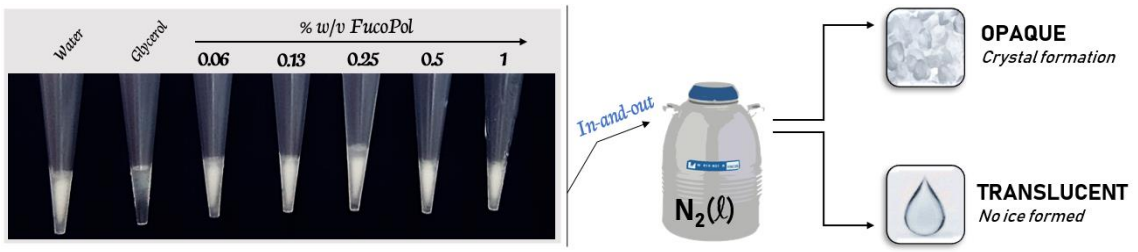


Figure IV.4. Schematic representation of the flash freezing assay and photographic evidence of the behavior of FucoPol in rapid cooling. Increasing concentrations of FucoPol were immersed and instantaneously removed from a -196ºC liquid nitrogen recipient and photographed. Distilled water and 50% v/v glycerol were used as negative and positive controls, respectively.

Whatever the concentration of FucoPol was, ice still formed and crystals grew to a considerable size just like in pure water. Glycerol was able to impede ice formation and the dim opaqueness most likely represents the limited growth of small ice nuclei that are kinetically entrapped in the viscous system, much like what happens in vitrification.

The flash freezing assay is a simple test that can be used to assess if a molecule can induce water to achieve a vitrified state. Glycerol and other polyol substances have for long been used in vitrification procedures and still are the unexpendable asset of cryopreservation. From a mechanistic perspective, immersion in liquid nitrogen causes a very fast cooling of the sample to cryogenic temperatures[169]. For small molecules and peptides, flash freezing is usually harmless, but for large proteins and supramolecular biological structures, including cells, it can be a very lethal procedure.

As water rapidly decreases in temperature, glycerol is able to maintain the solution in a supercooled state of matter, because its high viscosity kinetically hinders the re-orientation of water molecules to compose a crystalline matrix. As temperature drops further, the system is now in a metastable phase: although it is not a real state of matter, its boundaries lie between a solid and a liquid phase, where all molecules are in stasis – a glassy state.

Contrary to glycerol, FucoPol was unable to hinder ice formation and crystal growth at flash freezing rates. To further verify if FucoPol is definitely unable to achieve a state of vitrification, Differential Scanning Calorimetry (DSC) experiments were performed.

3.2. FucoPol is not vitrifiable but behaves like an antifreeze protein

The thermodynamic properties of FucoPol had already been explored by colleagues and it was reported that the biopolymer was thermostable above room-temperature[170]. Using a calorimetry technique, this experiment set out to explore thermodynamic behavior of FucoPol in harshly cold and hot environments.

Differential Scanning Calorimetry (DSC) assays can assess both the enthalpy (ΔH_x) of a phase transition and the temperature (T_x) – or range of temperatures – at which it is occurring. Because it involves cycles of heating and freezing a sample, both parameters for crystallization and melting events can be obtained. While T_c indicates both a water-to-ice phase transition and the influence a given substance has on the kinetics of crystallization, T_m can be a measure of crystal stability as it reflects the energetic probability of bond disruption.

The flash freezing assay provided evidence of ice growth. However, the thermodynamic properties of the crystals formed are governed by crystallinity, which is not a macroscopically quantifiable

property: flash freezing a sample might result in observing ice but gives no information on its stability, and this is usually assessed by T_m. A concentration range of 0.01–1% w/v FucoPol dissolved in deionized water both alone and in a mixture with proteins was tested and the data collected is presented in Table IV.3.

Table IV.3. Enthalpy and onset temperature data for the crystallization and melting events observed for all samples. ΔH and T represent the enthalpy and onset temperature, respectively, of either the crystallization (c) exothermic peak observed in the cooling cycle or the melting (m) endothermic band observed in the heating cycle. For cryopreservation purposes, analysis focuses on the onset temperature data with dH_2O as a reference sample for comparison. Data is an average of triplicates. Due to the stochastic character of crystallization, standard deviations are higher but the values are representative nevertheless.

Sample	ΔH_c (J/g)	ΔH_m (J/g)	T_c (ºC)	T_m (ºC)
dH_2O	-249.6 ± 34.4	293.9 ± 43.0	-18.7 ± 1.2	0.6 ± 0.1
BSA 1	-288.8 ± 3.0	342.3 ± 4.7	-17.8 ± 0.9	0.1 ± 0.1
AFP 1	-269.4 ± 22.8	314.2 ± 30.5	-16.6 ± 1.4	-0.4 ± 0.1
FP 0.1	-258.3 ± 33.5	307.1 ± 37.7	-18.4 ± 0.7	-0.3 ± 0.2
FP 1	-283.9 ± 7.6	336.8 ± 7.0	-17.8 ± 0.5	-0.6 ± 0.3
FP 10	-277.9 ± 23.1	317.6 ± 26.4	-13.1 ± 0.1	-2.6 ± 0.5
FP-BSA 1:10	-282.6 ± 4.1	340.5 ± 5.9	-19.5 ± 0.4	-0.5 ± 0.1
FP-BSA 1:1	-291.2 ± 4.4	340.8 ± 5.7	-15.8 ± 0.2	-0.5 ± 0.3
FP-BSA 10:1	-287.0 ± 4.1	339.7 ± 4.8	-16.9 ± 0.2	-1.7 ± 0.4
FP-AFP 1:10	-281.7 ± 7.7	335.1 ± 9.1	-18.2 ± 0.3	-0.2 ± 0.2
FP-AFP 1:1	-282.4 ± 0.3	341.0 ± 5.3	-19.7 ± 1.8	-0.3 ± 0.1
FP-AFP 10:1	-286.7 ± 18.1	340.2 ± 17.2	-15.1 ± 0.1	-1.1 ± 0.3

Besides assessing the individual contribution of FucoPol in the behavior of water when cooled and heated repeatedly, the biopolymer was compared to target references. This behavioral profiling of FucoPol allows to not just evaluate its antifreeze activity but also to classify it according to different modes of action.

To baseline the behavior of a non-antifreeze substance, BSA was used as a protein that is not antifreeze in nature. Its antifreeze counterpart was the HPLC6 type I antifreeze protein (AFP) produced by the arctic fish *Pleuronectes americanus*. This protein results from years of natural selection of species that were forced to survive at subzero temperature environments. It is a 37-residue protein with a single α-helical structure that interacts with the ice front of a forming crystal, hampering its growth[171]. This is known as the adsorption-inhibition mechanism and is based on the Gibbs-Thomson effect. Figure IV.5 represents a summarized representation of the thermodynamic events observed.

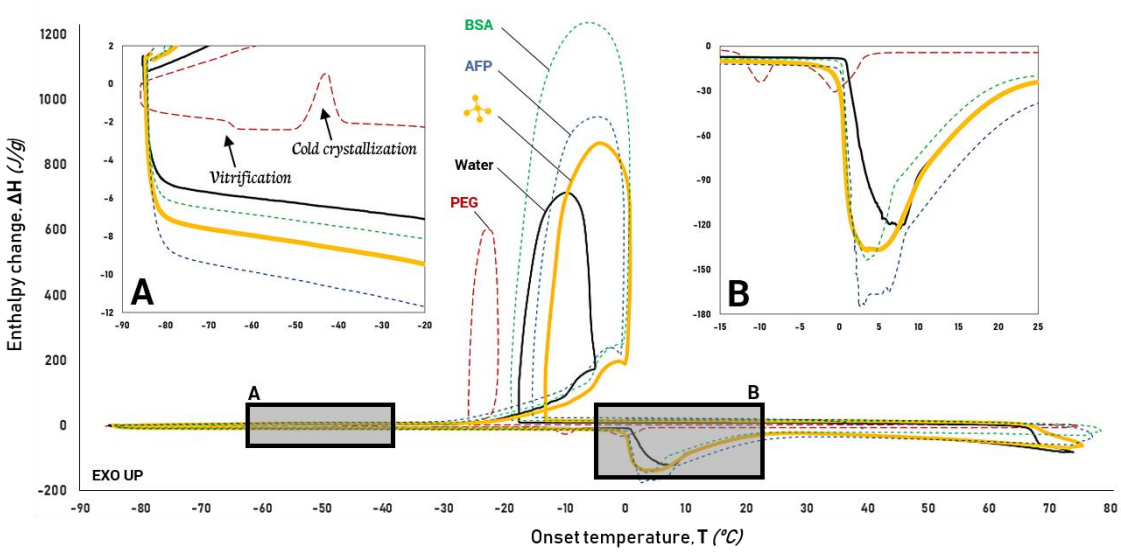


Figure IV.5. Summative multi-panel of the thermal analysis performed on several test solutions. In the center background panel lies the general representation of a DSC thermogram, with the crystallization peaks facing up (loop curve) and the melting bands facing down (panel B): hereby lies the single 1% w/v FucoPol solution representative of an antifreeze effect. Distilled water, 1 mg/mL BSA, 1 mg/mL AFP and additional 25% w/v polyethylene glycol (PEG) from unpublished data are also present as profiling references. Panel **A** shows the absence of any glass transition or cold crystallization events. Panel **B** shows the melting band broadening and a shift on the onset point to lower temperatures. Notice that the greater the deviation of the crystallization loop from the one of water, the higher the antifreeze effect, whatever may be the mechanism in question.

Before any discussion is carried out, it is important to note that in cryobiology the onset temperatures of crystallization (T_c) and melting (T_m) are the essential parameters of comparison. Enthalpy values are less significant in qualifying if a substance is antifreeze or not. The appearance of the thermograms presented and other minor characteristics are detailed in Section III.3.2 but are not crucial to the comprehension of the results obtained.

The crystallization phenomenon is usually divided into nucleation and growth, and nucleation can be (i) homogeneous when the nuclei are formed from rearranged water molecules or (ii) heterogeneous when an impurity is present and can itself act as the nucleus for ice growth. Distilled water presented an average crystallization temperature of -18.7 ± 1.2ºC and the ice formed melted at approximately 0.6 ± 0.1ºC. A crystallization temperature of -18.7ºC means that water was able to enter a supercooled state that started at 0ºC, but nucleation only occurred at -18.7ºC. Although regular tap water usually has overlapping T_c and T_m, this is due to the presence of impurities that act as heterogeneous nucleators.

After the temperature values for pure water were defined, any shift in T_c and T_m for the solutions tested would root from a direct contribution of a given substance to the phase transitions of water. To best understand the behavior of FucoPol, it is easier to first interpret the results obtained for BSA and AFP alone in order to understand what an antifreeze effect is. In the presence of 0.1% w/v BSA, there was no significant change in both event temperatures when compared to the presence of 0.1% AFP. With the antifreeze protein, T_c increased significantly to -16.6 ± 1.4ºC whilst T_m dropped to -0.4 ± 0.1ºC.

While it might appear that an earlier crystallization allows for more ice to form, the ice crystals formed are actually more segmented and smaller in size, so that when the temperature drops so

much that water molecules are unable to move, the frozen fraction has dimensions that are harmless in living systems (see Section I.2.2). A decrease in T_m indicates that the crystals formed are melting earlier. When a change in T_c is accompanied by a disproportional change in T_m, a non-colligative antifreeze mechanism is occurring[172]. Increasing concentrations of FucoPol resulted in a decrease in T_c up to a maximum of -13.1 ± 0.1°C and T_m also decreased to -2.6 ± 0.5°C, which is favoring a non-colligative mode of action and indicates that crystals formed are less energetically stable.

Unpublished data previously collected for 25% w/v polyethylene glycol (PEG) is also presented. The presence of PEG in solution results in cryoscopic depression (lower T_c) but T_m remains relatively constant, characteristic of a colligative mechanism. A decrease in T_c shows that the supercooled state has a longer lifetime and the system becomes vitrified. In panel A of Figure IV.5, vitrification is observed by a negative slope of the heat capacity of water (C_p) at *ca.* -65°C. Cold crystallization usually accompanies the glass transition and is a freezing phenomenon that occurs during melting as a consequence of regain of mobility, as water molecules can form transient crystals before melting once more[173].

It is clear that neither FucoPol nor the proteins tested are able to achieve a glassy state. The absence of vitrification is consistent with the observations done in the flash freezing assay. Although FucoPol is inadequate to vitrification solutions, its positioning in a cellular system is most likely extracellular due to its high molecular weight. Its mechanism of action would most likely be to shrink the cell due to an osmotic gradient and then providing protection against EIF.

To better visualize the temperature shifts induced by the presence of a given substance in water, a summary of their contribution to the postulated physical properties of ice is shown in Figure IV.6.

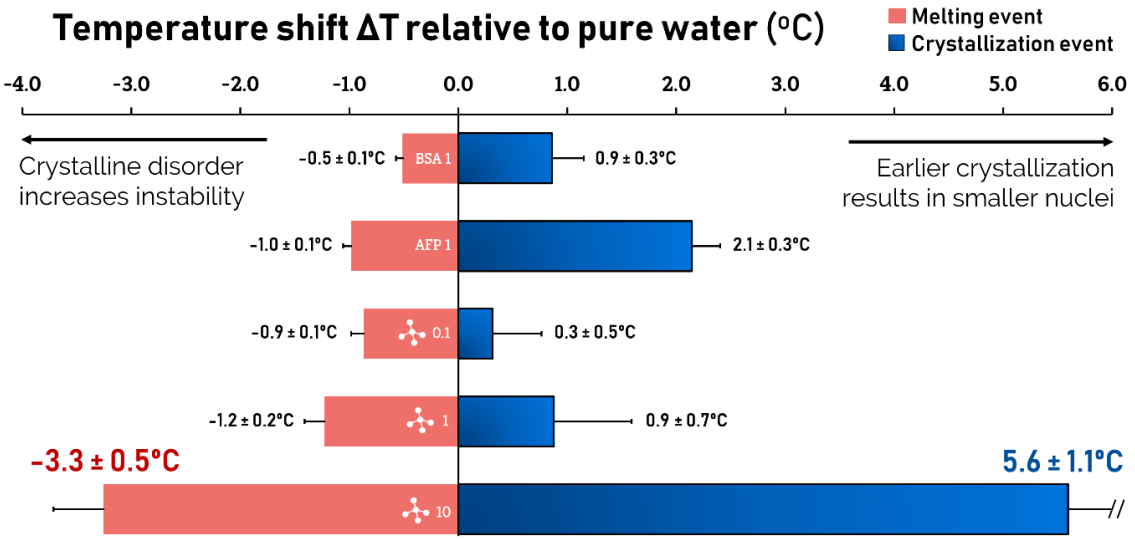


Figure IV.6. Column plot of the relationship between increasing FucoPol concentration and the physical properties of ice. Blue bars reflect the $Tc_{(sample)}$ – $Tc_{(water)}$ normalized value and an increase in bar length indicates that the onset of water crystallization in the presence of that sample has shifted to higher temperature values. Similarly, red bars reflect the $Tm_{(sample)}$ – $Tm_{(water)}$ normalized value but an increase in bar length indicates that the onset of ice melting has shifted to lower temperatures. Values were normalized considering a $Tc_{(water)}$ = -18.7 ± 1.2°C and $Tm_{(water)}$ = 0.6 ± 0.1°C. Notice that 1% w/v FucoPol seems to reduce both the amount of ice and its stability in the same fashion as an antifreeze protein.

A greater shift in T_c (to higher absolute values) indicates that crystallization is occurring earlier in the cooling cycle and is represented by a bigger blue bar. An increasing concentration of FucoPol yields a greater shift that eventually surpasses that produced by AFP. It is plausible that FucoPol might have a stretched conformation just like the α-helical AFP and therefore provides a barrier against ice growth. On the other hand, a greater shift in T_m (to lower absolute values) indicates that the melting of ice is occurring at lower temperatures, which was also observed in greater degree for 1% w/v FucoPol. Once again, FucoPol is thought to lead to the formation of fragile crystalline structures.

While crystallization shifts are a relatively direct interpretation of an antifreeze effect, the melting shift cannot be directly extrapolated to a decrease in crystallinity. A reduction of T_m with increasing concentrations of FucoPol could in fact be a misinterpretation of the reduction of the chemical potential of water: as ice gradually forms, the molar concentration of FucoPol increases (hypersaturation). As a consequence, a greater percentage of the unfrozen fraction of water is instead located in the hydration sphere of the biopolymer (which reduces entropy) and there is a lower drive for water molecules to be recruited to the ice front[174]. As a result, the melting point shifts to lower temperatures due to an increase in entropy and not because FucoPol is directly infiltrating the crystalline matrix.

The visual representation shown in Figure IV.6 reflects the magnitude of the contribution of a substance to the crystallization phenomenon but gives limited information on the mechanism of action of each substance. For example, it would appear that BSA has an effect similar to AFP because it increases T_c while decreasing T_m in the same fashion. However, BSA is not reported to be an antifreeze protein with non-colligative action.

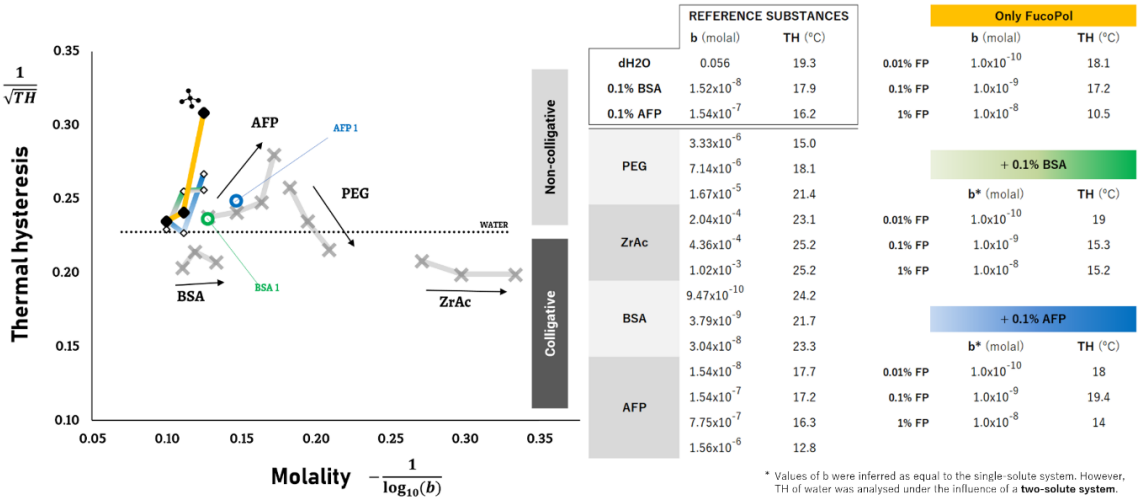
So far, it can be concluded that FucoPol behaves like an antifreeze protein, but to infer that it can simultaneously reduce the amount of ice formed while decreasing cristallinity, thermal hysteresis must be assessed.

3.3. Thermal hysteresis as a measure of antifreeze activity

Thermal hysteresis (TH) is defined as the temperature gap between crystallization and melting. In the case of ideally pure water, melting occurs at 0ºC but crystallization will occur at -42ºC if no nucleators are present[175]. An ideal nucleator would decrease thermal hysteresis to a point that $T_c = T_m$, which is the opposite of ice inhibitors.

Because thermal hysteresis often encompasses the concept of thermal history instead of a transiently independent state of a system, punctual temperature shifts are insufficient to define the antifreeze behavior of a substance. Instead, a thermal hysteresis plot defines the tendency a substance of varying concentration has to act in a certain way, and this is summarized in Figure IV.7.

Thermal hysteresis reflects not the molarity but the molality of a solute in water. Molality is a measure of concentration that has the advantage of only being dependent on masses of solute and solvent. Mass properties are unaffected by temperature and pressure variations and therefore provide an accurate concentration value to correlate to an antifreeze effect, which depends on relative mass and not on volume (avoiding hypersaturation-biased conclusions).



REFERENCE SUBSTANCES	b (molal)	TH (°C)
dH2O	0.056	19.3
0.1% BSA	$1.52x10^{-8}$	17.9
0.1% AFP	$1.54x10^{-7}$	16.2
PEG	$3.33x10^{-6}$	15.0
	$7.14x10^{-6}$	18.1
	$1.67x10^{-5}$	21.4
ZrAc	$2.04x10^{-4}$	23.1
	$4.36x10^{-4}$	25.2
	$1.02x10^{-3}$	25.2
BSA	$9.47x10^{-10}$	24.2
	$3.79x10^{-9}$	21.7
	$3.04x10^{-8}$	23.3
AFP	$1.54x10^{-8}$	17.7
	$1.54x10^{-7}$	17.2
	$7.75x10^{-7}$	16.3
	$1.56x10^{-6}$	12.8

Only FucoPol	b (molal)	TH (°C)
0.01% FP	$1.0x10^{-10}$	18.1
0.1% FP	$1.0x10^{-9}$	17.2
1% FP	$1.0x10^{-8}$	10.5

+ 0.1% BSA	b* (molal)	TH (°C)
0.01% FP	$1.0x10^{-10}$	19
0.1% FP	$1.0x10^{-9}$	15.3
1% FP	$1.0x10^{-8}$	15.2

+ 0.1% AFP	b* (molal)	TH (°C)
0.01% FP	$1.0x10^{-10}$	18
0.1% FP	$1.0x10^{-9}$	19.4
1% FP	$1.0x10^{-8}$	14

* Values of b were inferred as equal to the single-solute system. However, TH of water was analysed under the influence of a **two-solute system**.

Figure IV.7. Thermal hysteresis (TH) as a function of single-substance molality (b) for antifreeze behavioral profiling. Data collected in this work is colored: orange for FucoPol, green for BSA and blue for AFP. Unpublished data is shown in grey and the corresponding black arrows indicate the respectively tendency. The horizontal dotted line represents the thermal hysteresis of water as a reference. A green-gradient indicates an increase in FucoPol in presence of BSA, and the logic is identical for AFP with a blue-gradient. Notice that either upwards or downwards tendencies indicate antifreeze activity by non-colligative or colligative effects, respectively, whilst no change indicates lack of correlation between TH and b. To the right is a summative table of the raw data points used for graph construction. Error bars were hidden for visual convenience but values represent at least triplicate experiments.

Some characteristics of the plot presented in Figure IV.7 must be explained before any behavioral conclusions are taken. Plotting the inverse of thermal hysteresis against molality allows to interpret each substance in terms of their antifreeze activity (AA). As previously explained, a nucleator molecule will decrease TH while an inhibitor will cause an increase. An upwards tendency in Figure IV.7 with increasing molality indicates that a given substance is having an antifreeze effect in a non-colligative manner. A downwards tendency is nevertheless an antifreeze effect but in a colligative manner: this occurs when T_c decreases much faster than T_m (that is why it is considered that $\Delta T_m \rightarrow 0$).

In the previous section, the behavior of BSA was questioned to be similar to AFP due to erroneous interpretation. Figure IV.7 now shows that whilst AFP presents an upwards tendency characteristic of a non-colligative effect, there is no correlation between thermal hysteresis and BSA molality as TH hardly changes with a molality increase.

Zirconium acetate (ZrAc) has an interesting behavior that explains why thermal hysteresis can only be interpreted as an end result, masking several effects that influence its analysis. ZrAc is capable of inducing cryoscopic depression much like PEG, and the supercooled state is stabilized. T_m values also decreased with increasing molality, but whilst $T_{m,\,PEG}$ averaged at 0.1°C, $T_{m,\,ZrAc}$ averaged at 6.4°C, a much higher value that was interpreted as a stabilizing effect (data not shown). The end result indicates that ZrAc does not influence the behavior of water (lack of tendency) but is colligative ($TH_{ZrAc} < TH_{water}$), which seems counterintuitive. The stabilizing effect of ZrAc on T_m is the same for BSA that also showed an average T_m of 7.2°C at the highest concentration (data not shown).

In the light of this interpretation, FucoPol presents an upwards tendency characteristic of ice inhibition. Moreover, its profile is similar to the type I antifreeze protein which is known to act in a non-colligative manner[176]. After observing that FucoPol had a significant antifreeze activity, some

mixtures of FucoPol with BSA and AFP were prepared to assess the behavior of regular-antifreeze and antifreeze-antifreeze systems in an aqueous solution (still in Figure IV.7). First, it is important to assess how the antifreeze properties of FucoPol vary in the presence of albumin, the most common protein in blood plasma. Then, a mixture of FucoPol and AFP could provide a synergistic antifreeze effect, therefore potentiating a cryoprotective formula if implemented as a two-CPA system.

Notice that the contribution of FucoPol in the presence of either protein assumes an equal molality value to FucoPol alone (represented as b*). This is another advantage of molality: the molality of a single solute is independent of all others in a multi-solute solution. Therefore, while b represented the molal contribution of FucoPol to a water system, we can interpret b* as the molal contribution of FucoPol to a protein-water system.

Regarding the FucoPol-BSA mixture represented as a green gradient line, a 1:10 proportion of the substances results in an insignificant antifreeze activity, just like what was observed for 0.01% FucoPol (points overlap). However, the presence of BSA enhances the antifreeze activity of 0.1% FucoPol but increasing biopolymer concentrations have no increasing effect. In practical terms, this means that adding FucoPol to a formula that will be exposed to blood albumin in a 1:1 mass ratio will result in a potentiation effect of avoiding cryoinjury, which is very positive. Moreover, BSA does not have an inhibiting effect on the performance of FucoPol up to a 10:1 ratio, which corroborates the non-conflictuous interaction that was previously postulated.

The FucoPol-AFP mixture is represented as a blue gradient. From a 1:10 to a 1:1 ratio, both molecules are conflictuous with one another and result in a decrease of antifreeze activity. It is unknown why the system behaves like so, although is it thought that the adsorption-inhibition mechanism of AFP is less likely to occur when FucoPol is interacting with water as well. Anyhow, a 10:1 ratio results in an increase of antifreeze activity, characteristic of molecular synergy, although it does neither surpass the performance of FucoPol or AFP alone.

Polysaccharides that constitute the biofilm of a bacterial colony have been reported to present some degree of cryoprotection in general (see Table I.5). In summary, it is very likely that FucoPol can provide cryoprotection to living systems on its own and at relatively low concentrations. Despite its combination with AFP not yielding a greater effect, a non-nefarious interaction with BSA is a promising result in implementing FucoPol to perfusion formulas and protocols.

4. Cryopreservation assays

The core procedure on assessing the performance of a polysaccharide that possesses such promising properties is a cryopreservation assay. In ensuring that cell viability is maintained throughout a process of thermal stasis, several physical and chemical parameters must be taken into account for it to be a success. Besides mechanical damage arising from ice growth, metabolic arrest can cascade into a plethora of different harmful consequences and that is why all that involves a freeze-thaw procedure, from intricate technical handling to efficient carrier solutions, is so complex.

The cryopreservation assay was adapted by re-iteration and standardized to every procedure further presented in this work. A detailed scheme of the whole protocol can be found in Figure III.3. Notice that a relatively slow freezing rate of 1ºC/min was used, while thawing was fast. This might be counterintuitive, as slow freezing enables the growth of ice crystals before reaching a temperature in which molecules cannot overcome the kinetic barrier to re-organize. However, this

cooling rate has been widely used in cryopreservation protocols provided that a cryoprotective solution is present[177].

Experimentally, adverse conditions that maximize ice growth and damage will test solutions to their limit of cryoprotection, giving a realistic result of their performance.

4.1. *In vitro* assays require a sterile polysaccharide

In order to use any material in animal cell applications or any branch of biomedicine, it is essential that it is completely sterile. Therefore, this section provides an exploratory compendium on how to best sterilize FucoPol.

To achieve a state of sterility, six different methods were performed on FucoPol and are summarized in Figure IV.8. The presence of any microorganisms was assessed by resazurin: a color change from blue to purple was an indication of mitochondrial redox metabolism and the sample would be classified as contaminated.

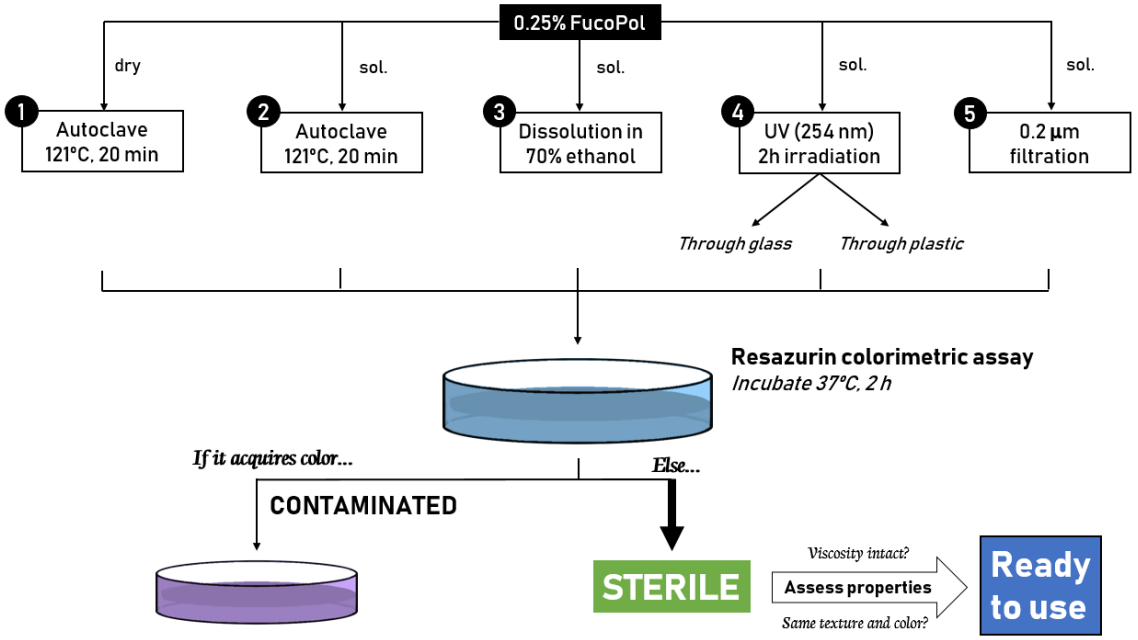


Figure IV.8. Schematic representation of the qualitative approach used in an attempt to reach sterility. Different approaches were used, either with FucoPol in a dry state (method 1) or in a 0.25% w/v liquid solution (methods 2-5). After each method, resazurin was added: a metabolic conversion to resorufin indicates presence of unwanted microorganisms and the method is considered to be ineffective. If sterility is reached, the Petri dish is additionally left to incubate for a week to guarantee complete sterility and after that period, the polymer had its apparent viscosity measured and contrasted with the original value prior to any sterilization treatment. If both the rheology and aspect of the polysaccharide were maintained, it was considered to be ready for use in the cell assays.

Essentially, a sterilization method is only reliable if it does not alter the original properties of the polymer, otherwise it is not useful even if proven to be effective for sterilization. Therefore, this experiment was purely qualitative and practical: if the polymer was not in a good condition, the method would be discarded without any comprehensive study.

The first approach (methods 1 and 2) was to use the default sterilizing method used in microbiology: autoclaving. FucoPol was both sampled in dry and aqueous solution states, and stored in Schott flasks. Both samples were sterile but the aqueous solution had a reduction in apparent viscosity. One possible explanation is the caramelization phenomenon. When glucose and sucrose are present, autoclaving a sample at high temperatures induces a non-enzymatic analog of the Maillard reaction, which is pyrolytic caramelization. Although these sugars have caramelization temperatures around 160ºC, impurities in the solution composition will decrease the temperature required for the reaction to occur[178]. In this basis, caramelization reactions are sensitive to the chemical environment and it could be inferred that dry-state polymers are less affected by Maillard reactions than aqueous solution but they could still occur to the extent of effectively altering the structure and properties of FucoPol.

A different approach was suspending FucoPol in 70% v/v ethanol (method 3), usually a germicide concentration. The beige cotton-like appearance of FucoPol turned to a brownish mesh of fibers. This visual observation was considered a negative outcome, so viscosity measurements or resazurin assays were not performed and the method was discarded.

In method 4, UV irradiation was performed on a 0.25% w/v FucoPol solution that was either in a glass or a plastic container. Assaying different containers had the intent of evaluating the penetrability of UV light through different materials. In both methods, FucoPol viscosity was unchanged but the samples were still contaminated and the method was discarded. It was thought that FucoPol could absorb UV light, therefore accidentally protecting the microorganisms, so a follow-up of this experiment was done and can be found in Section IV.8.5.

The last approach was to filter a polymer solution through a 0.2 µm filtering device, as it is usually done with several polymers. As there was no thermal or chemical treatment of the polymer, the rheological properties were intact and the FucoPol was effectively sterilized. However, there was a big loss of polymer content in the filter and the final concentration of FucoPol was lower (not quantified). Given that a known and exact polymer concentration is an important factor in formulating cryoprotective solutions, the method was also discarded.

In summary, autoclaving in a dry state proved to be the fastest, easiest and most efficient method for FucoPol sterilization. Given that the resazurin assay was only done for 2 h, the solution was left to incubate in resazurin for a longer period of time. Even after three months, the solution still had a blue, decontaminated color, ensuring that sterility was achieved with a functionally intact polymer.

4.2. The cytotoxicity premise

Whilst the first premise of FucoPol implementation in cryoprotective formulas was chemical inertia, the second is that the polymer must be biocompatible in the target biological system. Therefore, a standardized cytotoxicity assay was performed and the results are shown in Figure IV.9.

In this experiment, cells were simply exposed to the target substance at 37ºC and left to incubate to check if cells would die or proliferate. After 2 days of initial exposure, there was no evidence that cells were dying, so it was considered that the polymer was biocompatible. The concentration range studied covers the upper limit of solubility of FucoPol before a viscous heterogeneous suspension is formed (in-between 1–2% w/v), so the cytotoxicity assay was a very broad screening.

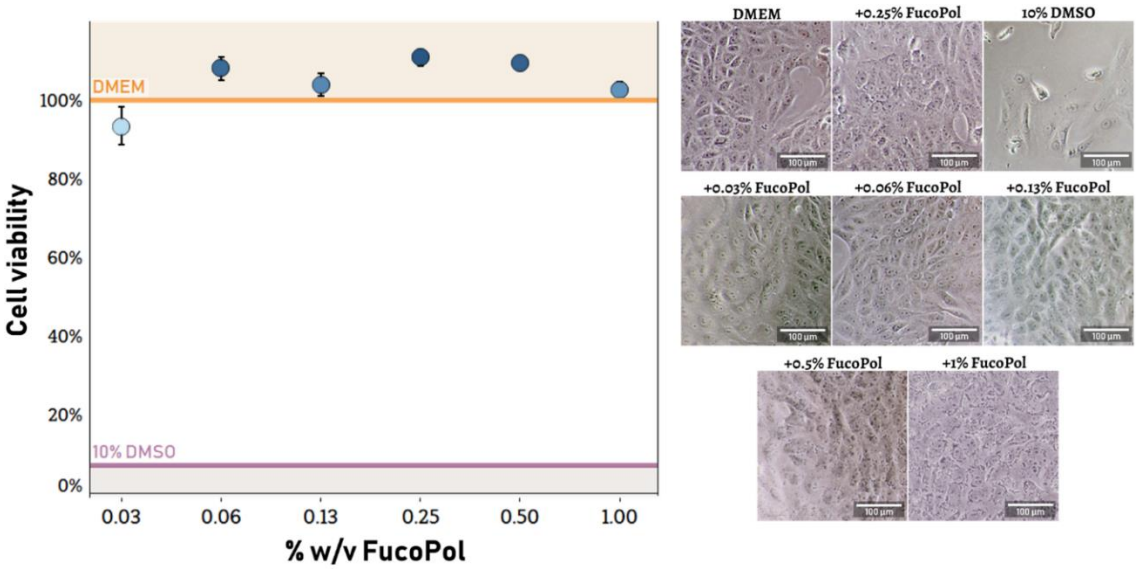


Figure IV.9. Cytotoxicity assay performed in Vero cells with FucoPol as the test substance. Increasing concentrations of FucoPol did not induce cell death as cell viability is close to that of exposure to DMEM. 10% DMSO was used as the positive control for cytotoxicity induction. On the right, morphological features are maintained in the presence of FucoPol. At higher concentrations, the cell surface is crowded with the polysaccharide but it does not seem to influence viability. The x-axis is a logarithmic representation. The cytotoxicity assay had a duration of 4 days.

Morphology was also assessed (Figure IV.9). Vero cells cultured in DMEM, which is the standard growth medium, presented a stretched aspect, indicating good adherence to the well. Their stretchiness is usually tolerant to the degree of cell confluence, so that when cell number is high, they become more compact but are nevertheless fully viable. In contrast, exposing cells to 10% DMSO alone for long periods of time resulted in cell death Neither solution formed which was confirmed by a spherical and loose appearance. In the presence of any given amount of FucoPol, cells maintained regular morphology and viability, with no indication of swelling, apoptosis, agglomeration or lysis.

Notice that instead of single-cell pictures, multicellular subsystems are presented because cell morphology is usually assessed considering cell-cell junctions. This last characteristic is particularly important in tissues and organs and must be preserved for correct function. Image coloring is irrelevant and not indicative of any unmentioned condition.

4.3. Can FucoPol secure viability in a freeze-thaw cycle?

Given that FucoPol is not conflictuous with cryopreservation solutions, has antifreeze activity and is not detrimental to cell viability, the first cryopreservation assay was carried out. It addressed both the ability of FucoPol to preserve cell viability after a freeze-thaw procedure and its stability in different ionic compositions and the results are shown in Figure IV.10.

The Vero epithelial cell line was routinely used throughout this work because it is the cell line suggested by the ISO 10993-5 policy for cytotoxicity testing.

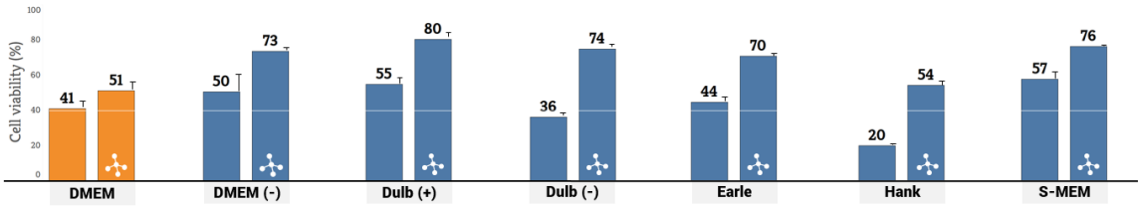


Figure IV.10. Post-thaw cell viability of Vero cells exposed to normothermic formulas of different ionic composition and supplemented with 0.25% w/v FucoPol. The transparent white line intersecting each viability bar corresponds to the post-thaw viability of VERO cells in the sole presence of DMEM as the control medium. Each data pair corresponds to a specific formula: the left and right bars indicate absence and presence of the polysaccharide, respectively. The value on top of each bar represents the corresponding observed viability. Notice the consistent increase in viability upon addition of FucoPol. DMEM = Dulbecco's Modified Eagle Medium; DMEM(-) = DMEM without FBS; Dulb(+) = Dulbecco solution; Dulb(-) = Dulbecco without Ca^{2+} and Mg^{2+}.

DMEM was used as the control medium to which FucoPol was added and is represented by the orange bars (Figure IV.10). Notice an increase in cell viability from 41 to 51% upon the addition of FucoPol, which corroborates the antifreeze activity previously documented. When FucoPol was added to normothermic formulas of different ionic composition (see composition in Table III.2), a significant increase in post-thaw viability was observed for all solutions tested. The cryoprotective effect can be easily assessed by the ratio of increase upon the addition of FucoPol. From left to right, it was observed a consistent increase of viability by 1.24, 1.46, 1.45, 2.06, 1.59, 2.7 and 1.33-fold.

These ratios allow to discuss two different topics. The first one is the contribution and adaptability of FucoPol to each composition tested. The biopolymer maintains its cryoprotective ability whatever the normothermic formula used and is consistently able to maintain post-thaw viability above the 70% mark for most solutions, at an average value of 68 ± 11%, compared to an average 43 ± 13% for unsupplemented formulas.

The second topic is a matter of synergy between FucoPol and specific ions. Although the sample array is quite heterogeneous, some tendencies can be reflected upon. In general, notice that while Vero cells are very tolerant to the different media, they adapt better to Dulbecco-type solutions, most likely a result of their similar composition to DMEM. Removing Ca^{2+} and Mg^{2+} from Dulbecco(+) decreases cell viability from 55 to 36%, but adding FucoPol to Dulbecco(-) is able to compensate that absence, resulting in 74% viable cells, compared to the 80% of supplemented Dulbecco(+). The presence of glucose does not seem to be crucial for cell viability, as Dulbecco-type solutions presented better results without this sugar (stock DMEM also had glucose but a worse outcome).

In assessing the contribution of a specific ion to a solution, a common issue prevents dissecting individual causalities from individual substances and is known as ion confounding[179]. Every ion is added in the form of a salt, which induces covariance when trying to vary an individual concentration. This can result in flawed experimental design, as preparing a formula using different salts from what is reported affects reproducibility. The normothermic formulas tested have multiple covariances that result in undistinguishable ion-specific effects. In the same fashion, any attempt at assigning ion contributions to the performance of FucoPol results in a very rough estimate.

In practical terms, cryopreserving Vero cells would yield the best results in FucoPol-loaded Dulbecco(+) for a maximum viability of 80% when compared with the regular 41% of DMEM. It is important to note that all solutions, DMEM included, contain 10% DMSO as a commonly used CPA. Therefore, it is very positive that FucoPol can synergize with DMSO in achieving better outcomes.

4.4. Cryoprotection is histologically universal

The added value of a polysaccharide increases proportionally with its spectrum of applications. It was mentioned before that cryoprotective formulas are often tailored to very specific tissues and organs, explaining the market diversity. Given that FucoPol demonstrated a significant cryoprotective ability for Vero cells, different cell lines were tested to assess if its behavior was independent of specific interactions with epithelial cells and the results are shown in Figure IV.11.

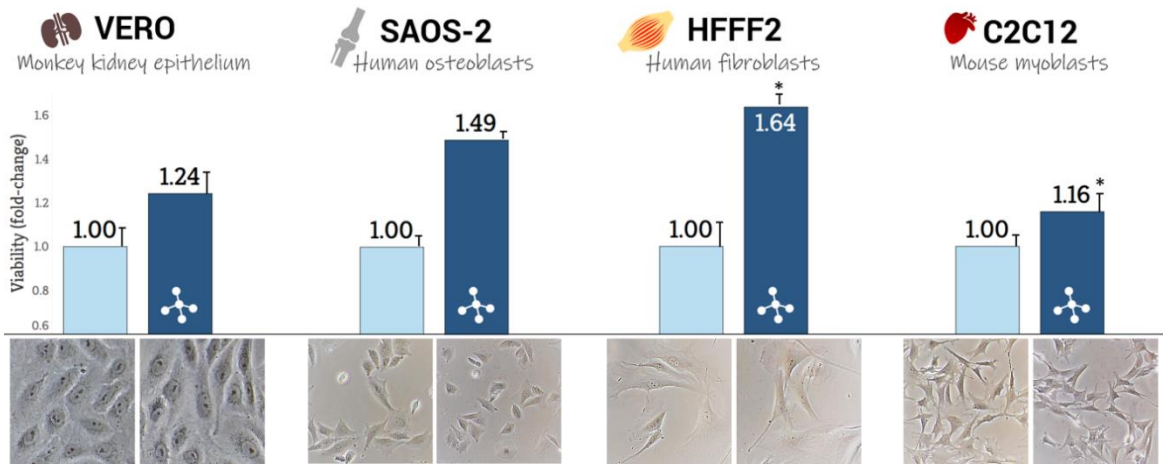


Figure IV.11. Post-thaw viability fold-change upon addition of 0.25% w/v FucoPol to different cell lines. The value on top of each bar represents the corresponding observed viability fold-change and is a ratio normalized to data set which was not frozen. Pairs of images represent each cell line in the absence (left) and presence (right) of the polymer. Notice that after freezing, cell morphology is maintained in both cases but the amount of salvaged cells differs.

A consistent increase of post-thaw viability was once again observed in the presence of FucoPol. All cell lines were cultured in their optimal culture medium and then exposed to FucoPol and frozen, yielding increases of 1.24, 1.49, 1.64 and 1.16-fold after thawing (Figure IV.11).

This ratio represents the quotient between the differences of viability post-thaw and at 37ºC when FucoPol is present and absent, respectively. Therefore, the results shown are normalized for the effect FucoPol has in the cells at 37ºC: if the biopolymer was cytotoxic to a given cell line, the fold-change ratio would be < 1.

Whilst some ratios appear greater than others, this might be a consequence of different growth rates. Every cell line was seeded at an equal concentration, but each cell has a different dimension, which could affect the rate at which they achieve confluence. Given this variability, it could appear that FucoPol would have little contribution to viability if freeze-related death was compensated by a greater growth rate. In general, Vero cells have an intermediate rate of growth, whilst Saos-2 and HFFF2 cells grow at slower rates, resulting in a greater viability gap between test conditions. With supporting evidence that morphology is unaffected by FucoPol in every cell line, the cryoprotective ability of FucoPol should remain relatively constant.

Given that Saos-2 derives from a malignant osteosarcoma, it was tested in the presence of FucoPol in the attempt to check how the biopolymer would interact with a cancer cell line. Unlike any other cell line, Saos-2 viability increased in its presence. In previous literature, it was shown that fucose-rich polysaccharides containing sulphate groups could prevent carcinogenic growth[180]. FucoPol does not contain sulphate moieties, but it could be potentially modified to ascertain a similar behavior. While the ability of cryopreserving cancer cells is still a desirable outcome to provide study resources to the field of experimental biology, it would be interesting to assess if that modification coupled with a cryopreservation assay would prove lethal to cells in both physical and chemical fronts or the two effects would nullify each other.

In summary, FucoPol does not show signs of cytotoxicity for heart, muscle, bone or kidney cells, which greatly increases its application range to different optimized formulas. Also, its cryoprotective ability seems to be independent of histology. Given that an antifreeze effect is directly correlated with the physical inhibition of ice growth, it should be a concentration-dependent phenomenon that does not seem to require extensive tailoring to different cell lines.

4.5. A novel polysaccharide approach: conjectures

So far it has been demonstrated that FucoPol can be efficiently used in cryoprotective formulas due to its broad-range stability, antifreeze activity and adaptability to different cell lines, greatly decreasing formula requirements of a specific biological sample.

However, the complexity issue is still a major aspect of successful cryopreservation. For example, normothermic formulas are designed to maximize cell growth and proliferation, but a hypothermic formula needs to play various roles in ensuring cell viability in the full range of the hypothermic continuum and back. Figure IV.12 succinctly summarizes the complexity leap required to ensure that cells have all the resources they need to survive.

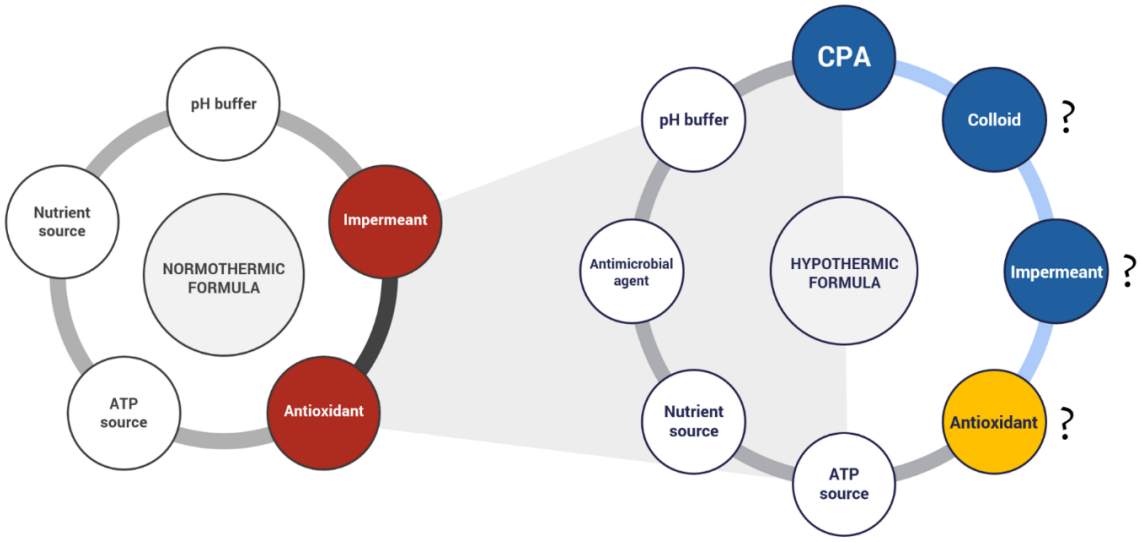


Figure IV.12. Schematic representation of the constitutive classes of normothermic and hypothermic formulas. There is an increasing complexity associated with ensuring cell viability when subzero temperature procedures are employed to a cell culture, because one has to consider that the hypothermic continuum is an unavoidable threat. Therefore, the difficulty associated with developing cryoprotective formulas requires a balance to be met between complexity (at the risk of implementing harmful substances) and high recovery rates.

The different classes of constituents all play a significant role in cell survival, the most important being the cryoprotective agent used. FucoPol has shown to efficiently contribute to this role, but some of its additional properties were suggestive of multiple functionality.

Thus, the concept of not only optimizing a cryoprotective formula but also simplifying its composition was an enticing idea. Therefore, it is hereby postulated what properties could instigate even better outcomes in biological cryopreservation:

- **Colloid agent:** due to the viscosity FucoPol imparts to aqueous solutions and its ability to maintain homogeneity of a solution a cryoprotective solution, it could potentially substitute dextran or starch-based solutions in a cryoprotective solution. FucoPol could act both as a CPA whilst ensuring that good solution homogeneity provides a balanced nutrient uptake in perfusion protocols.

- **Impermeant:** this class of components is usually populated by sugar compositions that regulate the osmolarity of a system from the extracellular space. The purpose of inducing a hypo-osmotic condition results in less freezable water to be present inside the cell at equilibrium, avoiding lethal cell growth to an extent. FucoPol has a molecular weight ranging around 1–5 MDa and its biofilm-like nature confers extracellular positioning. Therefore, instead of using a plethora of sugar mixtures, FucoPol could regulate osmotic balance, while still protecting the cell against ice damage from an extracellular position, where water is most abundant.

These postulations were directly derived from the intrinsic properties of FucoPol. Although some colloid and impermeant evidence stemmed from Section IV.2.1 and Section IV.4.3, respectively, they were not directly assessed with proper techniques. However, it is known that FucoPol contains *ca*. 25% (w/w) negatively charged acyl groups that at physiological pH could potentially scavenge oxidant species.

An antioxidant property is crucial to counteract the effects of metabolic waste accumulation, because cells cannot excrete metabolic by-products when frozen. This idea of simplification therefore required that an additional function ought to be observed in FucoPol besides its cryoprotective abilities. Previous work[150] had shown that antioxidants encapsulated in FucoPol had only a fraction of the total antioxidant potential observed, so we decided to test out its antioxidant properties alone.

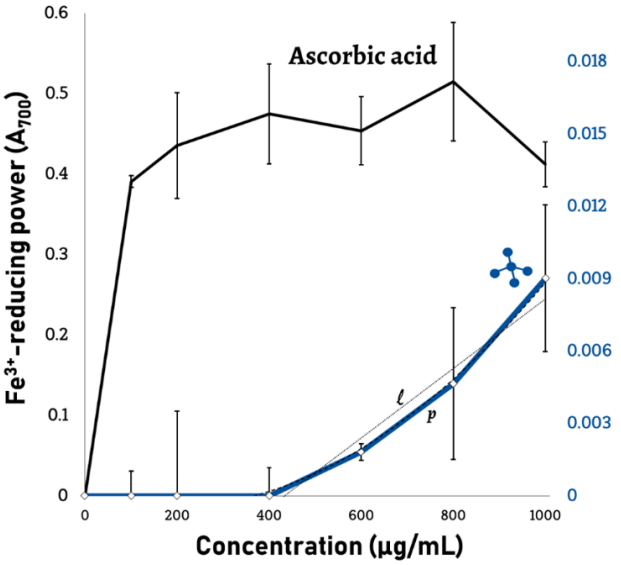
5. Antioxidant properties

In Section I.3.1, it was shown that the lethal mitochondrial cascade of events in the thawing stage can lead to cell death by oxidative damage. Hypothermic formulas can often be supplemented with chemical antioxidants like vitamin C, but these have actually shown to have a pro-oxidant effect[181]. Custodiol-HTK usually contains glutathione, a phase II enzyme of the liver detoxification pathway, as the sole antioxidant that increases cost of production. It was then of interest to assess the antioxidant properties of FucoPol and to evaluate its further benefit to avoid hypothermic continuum-related damage.

5.1. FucoPol scavenges oxidative Fe^{3+} species

Ferric reducing antioxidant power (FRAP) assays are commonly used to assess the scavenging properties of a given molecule. Simply put, an oxidant species is mixed with the study substance and the amount of reduced species is then quantified colorimetrically and its formation is interpreted as a direct causality from the activity of the target substance.

The first FRAP methodology used was initially pioneered by Oyaizu *et al.* and consisted of using $Fe(CN)_6^{3-}$ as the oxidant species[149]. FucoPol samples were prepared with concentrations ranging from 0.01 to 0.1% w/v in an attempt to define the minimal threshold for antioxidant activity, and the results are shown in Figure IV.13.



% w/v	Relative scavenging potential	
	AA	FP
0	0%	0%
0.01	75.9%	
0.02	84.6%	
0.04	92.3%	
0.06	88.2%	0.4%
0.08	100%	0.9%
0.1	80.1%	1.8%

Polynomial fit	$y = 1E\text{-}08x^2 - 5E\text{-}06x$	$R^2 = 0.9934$
Linear fit	$y = 1E\text{-}05x - 0.0066$	$R^2 = 0.9646$

Figure IV.13. FRAP assay using $Fe(CN)_6^{3-}$ as the oxidant species to assess the antioxidant power of FucoPol. Ascorbic acid (AA), known as vitamin C, was used as control and values were normalized to distilled water. Notice the dose-dependent increase of FucoPol in its ability to perform the Fe(III) → Fe(II) reduction. A minimal threshold is observed at 400 µg/mL, or 0.04% w/v. Linear (l) and polynomial (*p*) fits were done to extrapolate the antioxidant activity of FucoPol at working concentrations, with a second-order equation best describing its behavior. To the right is a table that summarizes all plotted data and corresponding experimental fitting. The scavenging potential is relative to the highest absorbance value observed for ascorbic acid.

FucoPol presented a dose-dependent increase of its activity to scavenge Fe(III) species. A minimal threshold was found to be *ca.* 0.04% w/v, which corresponds to 400 µg/mL, the common unit of comparison in antioxidant assays. Two fitting lines were drawn to interpret the behavior of FucoPol and a second-order polynomial was considered the best fit. However, the results obtained are located in a low concentration range and might not fully depict its behavior, as the linear fit also presents a 96.46% probability of being accurate, which is quite high.

There is an unusual appearance of the dose-response curve of ascorbic acid: the curve instantly jumps to near-complete reduction of Fe(III) species at the lowest concentration. Literature suggests that ascorbic acid reacts with Fe(III) species in a 1:2 stoichiometry[182], and this might explain the saturated appearance of the curve.

Nevertheless, when compared to ascorbic acid, the reducing power of FucoPol is still low. A mathematical approximation of binding kinetics yielded an EC_{50} of 18.7 µg/mL for ascorbic acid and 640.0 µg/mL for FucoPol. A direct visualization of the graph to assess at concentration corresponds to 50% activity yields an EC_{50} of 50 µg/mL for ascorbic acid and 750 µg/mL for FucoPol, which is still a considerable variation.

Calculating the EC_{50} value in this case – the effective concentration required to achieve 50% activity – is an incorrect approach as the antioxidant behavior does not follow a sigmoidal tendency. Usually, the EC_{50} value is based on a formula that uses a behavioral factor called the Hill coefficient:

$$\boldsymbol{y = (Bottom) + \frac{(Top) - (Bottom)}{1 + \left(\frac{x}{EC50}\right)^{-(H)}}}$$

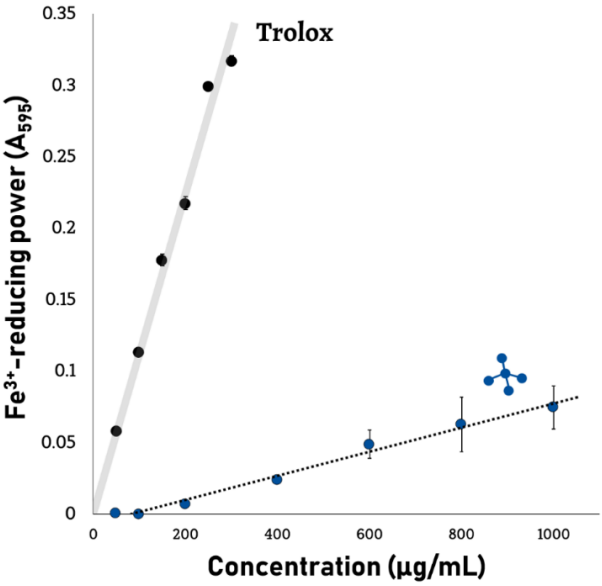
Where y is the observed experimental value, x is the corresponding concentration, Top is the highest observed value and Bottom is the lowest observed value. The Hill coefficient (H) is a value commonly used in biochemistry and pharmacology to reflect the binding nature of two substances and is a measure of cooperativity in dose-response curves[183]. To simplify the calculation, it was considered H = 1 (independent binding) as there is no indication that the binding of Fe(III) to FucoPol facilitates subsequent bindings, nor is the objective of this study to assess that.

Although the assay was performed using identical concentration ranges of both substances, their molecular weights differ and the amount of molecules of ascorbic acid present in solution is three orders of magnitude higher than FucoPol molecules. Although this may skew the results obtained, vitamin C is an antioxidant of excellence and the study has enabled to confirm that FucoPol has a measurable reducing power and might supplement hypothermic formulas probably as a secondary antioxidant, but not as the sole antioxidant.

Given that the working FucoPol dosage in cryopreservation assays was 0.25% w/v FucoPol, the experimental fittings performed on the data allow to extrapolate an antioxidant activity value of 0.02–0.05 absorbance units, which is 2.2 to 5.6-fold higher than the highest concentration value tested. Interestingly enough, this appears to be very close to the scavenging potential plateau for ascorbic acid and is an indication that FucoPol may be able to efficiently protect cells from oxidative damage.

The uncertainty of measurement demonstrated this assay to be of low sensitivity at the concentration range used. A possible explanation for the huge error bars obtained is that residual redox activity greatly influences data collected at 700 nm. Nevertheless, the obtained results were unsatisfactory and required validation, so a new FRAP assay that used Fe^{3+}-TPTZ as the oxidant species was used and the results obtained for similar concentration ranges but using Trolox, a water-soluble analog of vitamin E, as the reference, are shown in Figure IV.14.

Once again, a dose-dependent increase of the scavenging potential was observed and the minimal threshold located between 200 and 400 µg/mL. This seems to be a consistent observation in antioxidant assays using high molecular weight polysaccharides (see Table IV.4) and might reflect their sensitivity to oxidative environments.



% w/v	Relative scavenging potential	
	Trolox	FP
0	0%	0%
0.005	18.3%	
0.01	35.7%	
0.015	56.1%	---
0.02	68.6%	2.2%
0.025	94.2%	---
0.03	100%	---
0.04	---	7.6%
0.06	---	15.4%
0.08	---	19.8%
0.1	---	23.5%

Trolox linear fit	y = 0.0011x	R^2 = 0.9836
FP linear fit	y = 8E-05x - 0.0069	R^2 = 0.9876

Figure IV.14. FRAP assay using Fe^{3+}-TPTZ as the oxidant species to validate previous results on the antioxidant power of FucoPol. Trolox, a water-soluble analog of vitamin E, was used as control and values were normalized to distilled water. Once again, a dose-dependent increase is observed and the antioxidant potential starts to be significant in the 0.04% w/v region. To the right is a table that summarizes all plotted data and linear fits for further extrapolation are shown for both substances. The scavenging potential is relative to the highest absorbance value observed for Trolox.

Approximated EC_{50} values were calculated once again: using the Hill adapted formula, Trolox has an EC_{50} of 90.8 µg/mL while for FucoPol it is 318.4 µg/mL. Visual observation of the graph directly yields an EC_{50} value of 100 µg/mL for Trolox and 500 µg/mL for FucoPol. The linear fit obtained for FucoPol also allowed to extrapolate 0.19 units of scavenging potential at 0.25% w/v FucoPol, which is the equivalent to using 176 µg/mL of Trolox. Table IV.4 shows a summary of the results obtained and comparison with available literature.

Notice that EC_{50} values are substrate-specific and vary in different assays, because redox reactions are based on chemical potential that depends on the chemical environment surrounding FucoPol. The EC_{50} values obtained using the adapted formula were arbitrarily compared to direct visualization of the graph. In the $Fe(CN)_6^{3-}$ assay, the scavenging potentials calculated using both methods diverged 2.3-fold, while in the Fe^{3+}-TPTZ, the divergence was 1.4-fold. Despite the low accuracy of visual interpretation, this variation allows to question the usage of H = 1 as an approximation of binding behavior, but further studies are needed to assess how FucoPol binds to oxidative species.

Once the antioxidant activity of FucoPol was validated, a scale-up to an *in vitro* system would definitely substantiate these claims if the biopolymer could protect cells from oxidative damage.

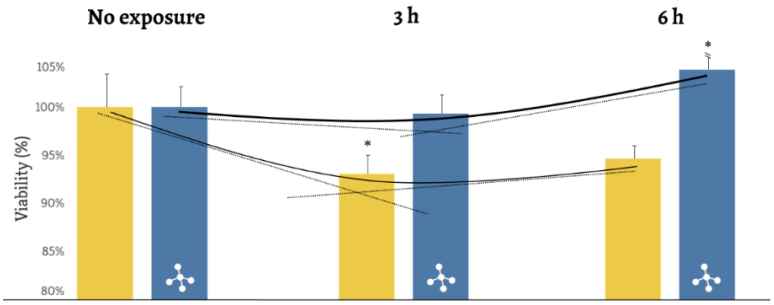
Table IV.4. Literature collection of antioxidant properties observed in polysaccharides from different sources. There seems to be a consistent observation that antioxidant activity starts at 400 µg/mL. Different experiments represent different sources of data that are not fully translational. Fe^{3+} reduction assays refer to contrasts with ascorbic acid, unless referred otherwise.

Polymer	Source	Assay	Approximate EC_{50}	Ref.
Glucose and rhamnose-rich EPS (10^5 Da)	*Weisella cibaria* GA44	DPPH Fe^{3+} reduction $O_2^{\cdot-}$ scavenging $OH^{\cdot}$ scavenging	4000 µg/mL 2000 µg/mL 1200 µg/mL 900 µg/mL	184
Starch-like EPS (10^4 Da)	*Peanibacillus mucilaginosus* TKU032	DPPH Fe^{3+} reduction	157.1 µg/mL Undetermined, max. 44%: 500 µg/mL	185
Fructose-rich EPS (10^6 Da)	*Paenibacillus polymyxa* EJS-3	Fe^{3+} reduction $O_2^{\cdot-}$ scavenging $OH^{\cdot}$ scavenging	Undetermined, max. 44%: 1000 µg/mL 400 µg/mL 220 µg/mL	186
FucoPol	*Enterobacter* A47	Fe^{3+} reduction	750 µg/mL (ascorbic acid) 500 µg/mL (Trolox)	This work

5.2. FucoPol potentiates cell recovery after exposure to ROS

Cells that are thawed from a frozen system suffer mitochondrial membrane disruption that leads to cytosolic overload of ROS, as detailed is Section I.3.1. This is one of the fundamental reasons why cell viability is only assessed 24 hours after thawing the cells, as necrotic and apoptotic cascades have been shown to occur in the first 6 h of post-thaw metabolism[187].

Once the antioxidant activity of FucoPol was demonstrated in the previous section, an *in vitro* experiment the oxidative damage that occurs after thawing. In this assay, cells were frozen and thawed as usual, but kept at 37ºC and exposed to H_2O_2 for different time periods. The presence of H_2O_2 allows to produce an experimentally controlled system of oxidation that forces cells to battle against oxidative damage. The full mechanism is detailed in Section III.5.3 and the results obtained for different exposure times in the presence of FucoPol are shown in Figure IV.15.



Exposure time	Control	+FP
0 h	100%	100%
3 h	93%	99%
6 h	95%	104%
24 h	97%	99%

	Drop rate	Recovery rate
Control	$y = -\frac{7}{3}t + 100$	$y = \frac{2}{3}t + 91$
+FP	$y = -\frac{1}{3}t + 100$	$y = \frac{5}{3}t + 94$

Figure IV.15. Viability of Vero cells exposed to 300 µM H_2O_2 for different periods of time at 37ºC. Yellow and blue bars indicate the absence or presence of 0.25% w/v FucoPol, respectively. Putative behavior lines were drawn for clarifying how the cells react differently to ROS-mediated oxidation and are shown in black. The point of inflection, or *turn-over point*, is represented by the intersection between the thin dotted lines. To the right is a table that summarizes the plotted data, with an additional 24-hour mark which was not represented. To quantify viability loss and recovery over time, linear equations of the dotted lines were determined for both DMEM and DMEM+FP. Notice that in the presence of FucoPol, the drop rate is smaller whilst the recovery rate is higher. The y-axis only spans the upper fraction of viability for visual convenience.

Note that before any interpretation is done, cells were cultured in DMEM which contains a significant amount of antioxidants. It is expected that these supplements are contributing to

nullifying the effects of H_2O_2, however that effect is normalized for all test conditions. Also, it is unknown if FucoPol has any enhancing or inhibiting interactions with those substances, but conclusions taken were based on the end result, giving little importance to these details.

In the control medium, the viability dropped when cells were exposed to H_2O_2 and increased slightly after 6 h. In the presence of FucoPol, it is possible to observe that not only viability did not decrease as much after 3 h, but growth was faster. Morphology is not shown as cells did not present visible changes, either in the presence of absence of FucoPol or at different exposure times: the oxidative consequence appears to only affect cells at a metabolic level in its initial stage.

FucoPol demonstrated an *in vitro* antioxidant effect, although the mechanism remains for elucidation. Two hypotheses can be postulated: either we are in the presence of (i) a direct scavenging effect, as FucoPol is able to interact with the ROS produced and self-oxidate or (ii) both a direct scavenging and an additional indirect interaction with other antioxidants in the system, enhancing the antioxidant consortium of the cell to battle the threat more efficiently.

Cell growth rates are also affected by exposure. Drop and recovery rates were roughly estimated for cells exposed to H_2O_2 in the presence or absence of FucoPol, and their intersection defines a *turn-over* point for cell homeostasis. When FucoPol is absent, the drop rate is 7-fold higher than when 0.25% w/v are supplemented. Similarly, recovery rates see a 2.5-fold increase in its presence. Curiously enough, the *turn-over* point is *ca.* 3 h for both conditions. While this value may be insufficient to conclude anything on stress defense mechanisms, it seems that the way FucoPol protects cells against oxidative damage is not correlated with any kinetic delay but with a direct scavenging of ROS.

To conclude, FucoPol is actively counteracting the acute oxidative damage cells suffer in the presence of H_2O_2 and further aids in their recovery and proliferation after regaining homeostatic balance. An additional measurement after 24 h of exposure was performed to assess if a long-term mechanism could arise and induce cell death even in the presence of FucoPol, but that was not observed. It is uncertain what outcomes would FucoPol self-oxidation induce in its ability to protect cells, but the results after 24 h do not indicate any significant nefarious effect.

This *in vitro* experiment is supporting evidence that an antioxidant effect could induce cell metabolism to bypass lethal apoptotic cascades. Although cells still undergo some stress, the overall aftermath is one where an organ could avoid total failure and be viable for transplantation. Table IV.5 summarizes a collection of experimental data from literature that corroborates the results obtained.

The ability to provide protection against physical and chemical injury, both as a cryoprotective agent and an antioxidant without requiring concentration increases is a promising result and shows that FucoPol can be a multifunctional approach for the tailoring of cryoprotective formulas.

Table IV.5. Literature data obtained for a FucoPol-like polysaccharide regarding antioxidative outcomes. An adapted compilation of different assays is presented and ranges different approaches to answering a

common problem. Notice that the obtained *in vitro* ROS scavenging results can be cross-correlated with more complex assays that corroborate anti-apoptotic behavior.

Source	Assay	Results of adding a fucose-rich EPS	Ref.
***Bacillus megaterium* RB05**	$OH^{\cdot}$ scavenging	EC_{50} = 200 μg/mL	151
	H_2O_2 scavenging	EC_{50} = 1500 μg/mL	
	Singlet O_2 scavenging	EC_{50} = 100 μg/mL	
	$O_2^{\cdot-}$ scavenging	EC_{50} = 25 μg/mL	
	DPPH	EC_{50} = 75 μg/mL	
	$ABTS^{+}$	EC_{50} = 275 μg/mL	
	β-carotene	EC_{50} = 25 μg/mL	
	H_2O_2 exposure	Cell distortion over time was averted, morphology maintained	
	G_0/G_1 cycle progression	Halted mitotic cycle was continued, cells left sub-G_0/G_1 stage	
	H_2O_2-induced apoptosis	Apoptosis is avoided, necrosis is also reduced	
	H_2O_2-induced intracellular ROS	Intracellular ROS is reduced by direct scavenging	
	Mitochondrial function	Membrane potential recovers faster Bcl-2 anti-apoptotic proteins are less expressed Bax/Bcl-2 proteins were downregulated at the mRNA level	
	Apoptotic inhibition	Cytochrome C can translocate back to the mitochondria Caspases were downregulated and PARP was cleaved Polymer synergizes with caspase-specific inhibitors	
	Cellular signaling	Nrf2/Keap1 anti-apoptotic pathway inactive due to ROS absence	

6. Combinatorial substitution: tailoring cryoprotective formulas

FucoPol has shown proficiency in both protecting cells against cryoinjury and oxidative damage. However, true formula simplification could only be achieved if we consider that multiple properties are observed simultaneously without the need of additional supplementation. Therefore, the following assays comprised an extensive screening on hypothermic formulas by substituting one or more constituents while simply adding the usual 0.25% w/v of FucoPol. Figure IV.16 presented below depicts an experimental strategy to assess the adaptive performance of the biopolymer to the absence of essential constituents, hereby named combinatorial substitution.

The two methods used in the combinatorial substitution experiment were knock-out and gradient-based substitutions. They provide different interpretations to the same hypothesis and are fully detailed in Section III.6.1. In Figure IV.16, the pathway of a commercial solution X represents the knock-out methodology, in which one substance was removed while FucoPol was added in a one-step substitution.

The resulting viability from comparing this test condition to the control formula enables to assess how much of a compensation can the biopolymer provide to the absence of a constituent. Then, incremental removals were performed, and the final solutions still contain 0.25% w/v FucoPol, which enables to quantify how many constituents can FucoPol substitute. In the case of commercial solution Y, the focus is on a single class of constituents. The addition of FucoPol is accompanied by a gradual decrease of that class to the point of total absence, and this strategy allows to define (i) how essential that constituent is to the original formula and (ii) the adequate FucoPol:constituent ratio that yields the most benefit.

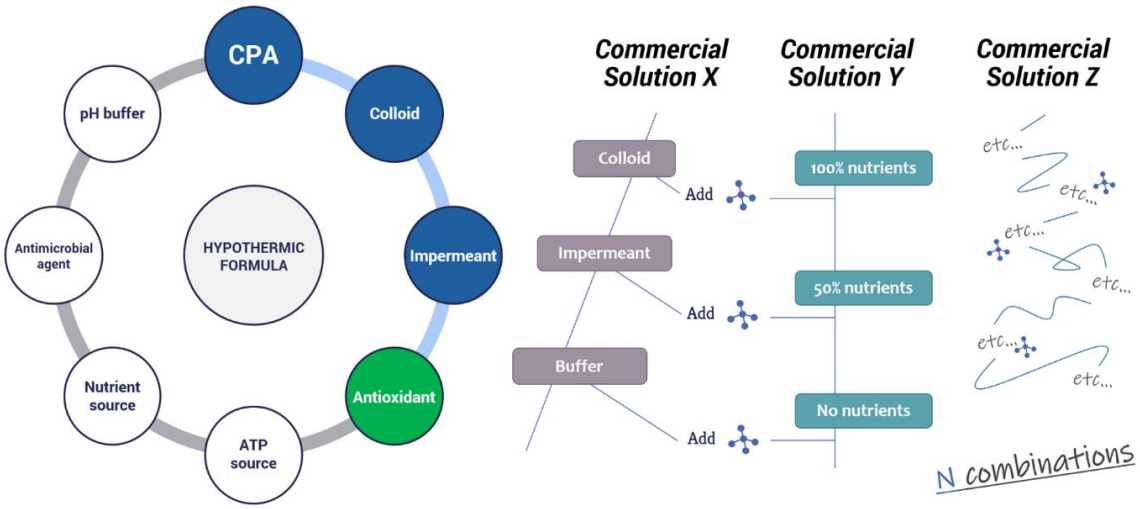


Figure IV.16. Schematic representation of the complexity of formulas tailored to protect biological samples from the injurious hypothermic continuum. Amongst all other properties of FucoPol and after verifying its antioxidant potential, there are several experimental approaches to assess if the polysaccharide can substitute others constituents in hypothermic formulas. Either by knock-out (example X) or gradient-based (example Y) substitutions, a stepwise analysis of the contribution of all substances can be assessed in the path to optimization.

Table IV.6. Summary of all substitutions performed in hypothermic formulas. For each condition tested, the addition and removal of each constituent is reported. The addition FucoPol for a final concentration of 0.25% w/v in each condition (S1, S2, S3) is implicit and therefore not shown. Additionally, an economic evaluation of the initial and costs for each formula were calculated in order to assess any marketable advantage, along with the number of original constituents removed and average post-thaw viability. The prices were calculated using Sigma-Aldrich reagents and reflect the cost of producing 1 L of solution. FucoPol was considered to have a cost of production ranging from 5 to 1500€/kg. Shorter names for the formulations were adopted for convenience.

	C	S1	S2	S3
EC	*see Table III.4*	Glucose reduced to 50%.	Glucose removed.	
	Initial price: 23€ Viability: 48%	Final price: 18–22€ Substitutions: 0.5 Viability: 64%	Final price: 14–18€ Substitutions: 1 Viability: 71%	
Unisol*	*see Table III.4*	Gluconate and Dextran-40 removed.	Equal to S1. NaH_2PO_4/Na_2HPO_4 buffer substituted by HEPES.	Equal to S2. Sucrose and mannitol removed.
	Initial price: 160€ Viability: 80%	Final price: 4–8€ Substitutions: 2 Viability: 65%	Final price: 12–16€ Substitutions: 4 Viability: 58%	Final price: 10–14€ Substitutions: 6 Viability: 75%
Custodiol	*see Table III.4*	---	Mannitol removed.	
	Initial price: 2€ Viability: 43%	Final price: 2–6€ Substitutions: 0 Viability: 66%	Final price: 1–4€ Substitutions: 1 Viability: 38%	

*The sole source of K^+ in Unisol-CV comes from the addition of 100 mM potassium gluconate. Gluconate was removed in all substitutions. To avoid overinterpreting the absence of gluconate due to a disrupted Na/K ratio, all test samples were compensated by adding 70.69 mg K_3PO_4. Also, Na^+ deficits arising from substituting the phosphate buffer were compensated by adding 27.38 mg Na_3PO_4 to S2 and S3.

Despite the immense amount of combinations that can be performed, this work focused on substitutions that targeted specific functions to be assessed for FucoPol. However, a scale-up of assessing almost all combinations possible is a promising step in building an interactive network

that spans many substances and not only FucoPol.Then, by patterning specific interactions, a predictive model can be built to assess which substances work best when used together or even design new molecules with desired properties. Table IV.6 summarizes the substitutions performed on three hypothermic formulas and resulting viabilities obtained for these transformations.

6.1. Hypothermic formulas: the concept of multifunctionality

Of all combinations tested and presented in Table IV.6, this section gathers three study cases for interpretation of what to expect from a combinatorial substitution screening. Case 1 is a knock-out elimination method performed on Custodiol-HTK. Case 2 is performed on Euro-Collins, but unlike case 1, it is a gradient-based elimination method. And finally, case 3 shows incremental knock-out eliminations on Unisol-CV. For the original composition of each formula, refer to Table III.4.

Custodiol-HTK is a low-viscosity formulation initially designed for open heart surgery perfusion in the 1970s. Nowadays, it is used in the preservation of liver, kidney and pancreas but high volumes are used (a waste of as much as 5 L per organ). Contrary to other hypothermic formulas, it is an ICF-type solution, as the low potassium is designed to reduce the K^+-mediated vasoconstrictive effect during organ thawing and washout[188]. Figure IV.17 shows the results obtained for case 1.

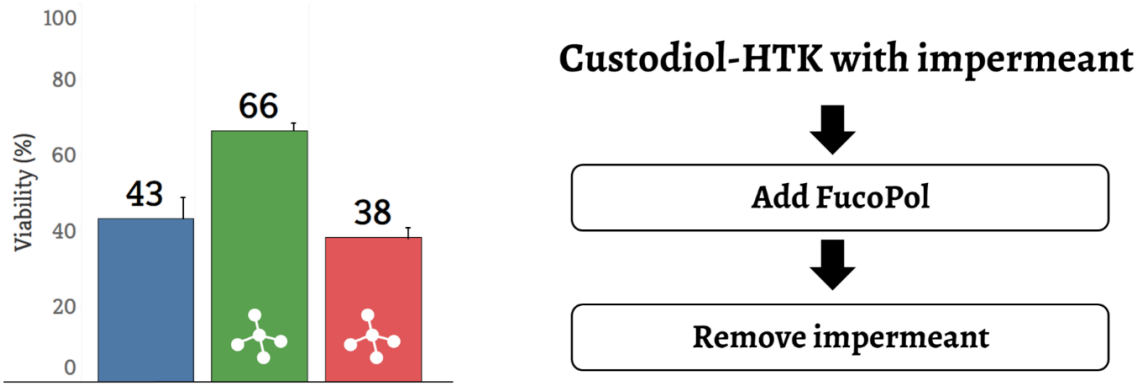


Figure IV.17. Post-thaw viabilities obtained with the knock-out substitutions performed in Custodiol-HTK. The right panel describes the experimental sequence (in order: blue, green, red) and values on top of each bar represent the average post-thaw viability for each condition. After removal of mannitol, FucoPol was not able to fully compensate its absence.

The original composition of Custodiol-HTK contains 30 mM mannitol as the sole osmotic regulator and yielded a post-thaw viability of 43 ± 5%. When FucoPol was added to its original composition, there was a 1.5-fold increase in the number of viable cells, but the sole removal of impermeant resulted in a drop to 38 ± 2% even in the presence of FucoPol. A synergistic effect is observable when both substances were present, as it yielded the highest viability. However, using just one of either substance decreased viability to similar viability values: mannitol proved to be slightly better in this specific composition, although viability values are still lower than expected.

Low Na^+/K^+ contents in Custodiol-HTK is a possible explanation for lower cell number, as perfusion times are lower than the incubation procedure performed. This experiment allows to conclude that this hypothermic formula was designed to synergize very well with mannitol, so it proves to be an invaluable resource to the medium. While it is unwise to completely elimination mannitol for practical use, it must be emphasized that the addition of FucoPol to the original solution actually resulted in a much better outcome, yield 66 ± 2% cell viability.

Euro-Collins was one of the first cryoprotective compositions to have been produced in 1980, when a high glucose content was added to the Collins composition (originally created in 1969), in order to serve an impermeant function, reducing cold ischemia injury in kidney, heart, liver and lung grafts[42]. Figure IV.18 shows the results obtained for case 2.

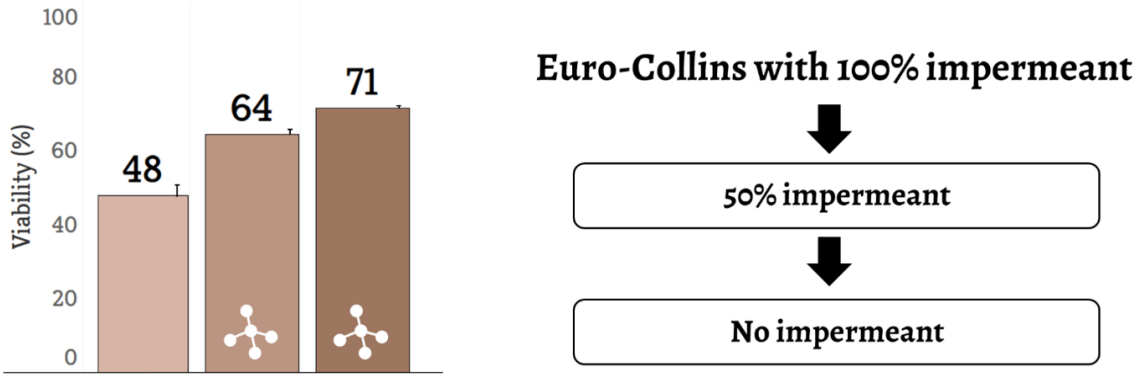


Figure IV.18. Post-thaw viabilities obtained with the gradient-based substitutions performed in Euro-Collins. The right panel describes the experimental sequence (in order: light, medium, dark brown) and values on top of each bar represent the average post-thaw viability for each condition. Gradually removing glucose, which acts as an impermeant in this formula, and adding FucoPol in compensation seems to increase its performance.

Once more, the analysis focused on impermeant molecules. However, instead of completely knocking out glucose, a gradual two-step removal was performed. The original composition yields 48 ± 3% post-thaw viability. This value increases 1.3-fold when FucoPol is added and glucose reduced to 50% w/v and 1.5-fold when glucose is completely removed, relying solely on FucoPol for osmotic regulation.

One curious aspect of this experiment is that whilst excess glucose aids in avoiding cryoinjury (due to higher viscosity) and serves an impermeant purpose, it can also penetrate the cells to provide energy from glycolysis. It is possible that 194 mM glucose are sufficient to avoid starvation-related cell death, so it was assumed that FucoPol was compensating for the loss of osmotic regulation.

Case 2 proved to be very promising as it showed a successful substitution of a constituent accompanied by viability increase in its absence. Also, Euro-Collins has an approximated manufacturing cost of 23€, and this number was reduced to an average of 16€ in the sole presence of FucoPol. To conclude, FucoPol was able to compensate the elimination of glucose, which was a major constituent of the formula.

Lastly, **Unisol-CV** is a hypothermic formula designed by Organ Recovery Systems Inc. for experimental optimization in endothelial cell lines[152]. Of all hypothermic formulas tested, it is the most complex solution in terms of impermeant and colloid classes. Figure IV.19 shows the results obtained for case 3.

This specific study had greater risk associated, as the knock-out eliminations were incremental. As a result, interpretation became more intricate but was necessary to assess the full potential of FucoPol.

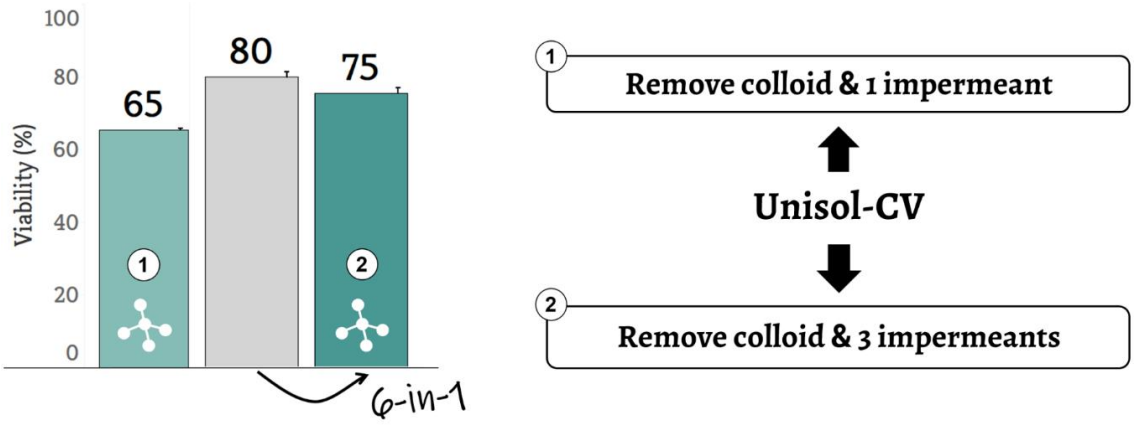


Figure IV.19. Post-thaw viabilities obtained with the knock-out substitutions performed in Unisol-CV. The right panel describes the experimental sequence and values on top of each bar represent the average post-thaw viability for each condition. Notice that a trade-off of six extracellular constituents for 0.25% w/v FucoPol alone yields 75% viability, which is very similar to 80% of the control solution.

The original composition of Unisol-CV resulted in 80 ± 2% post-thaw viability, which is an acceptable value in realistic situations. Upon the removal of gluconate (impermeant) and dextran-40 (colloid), which corresponds to the Unisol-S1 condition (see Table IV.6), viability dropped to 65 ± 1% even when FucoPol was supplemented (1). Although it is possible that FucoPol might have attenuated the drop in viability from the removal of two constituents, the result was not positive from a biomedical point-of-view.

However, incremental removals were continued: when gluconate, dextran, sucrose and mannitol were removed, phosphate buffer was exchanged for HEPES and FucoPol was added, which corresponds to the Unisol-S3 condition, 75 ± 2% viability was obtained (2). Although it might be enticing to conclude that the substitution was a failure, notice that a total of 6 constituents were removed and FucoPol was left to compensate for their absence.

From a different perspective, when 144 mM worth of backbone substances were removed from Unisol-CV, 0.25% w/v FucoPol was able to guarantee a similar performance within a 3.5% error of its original performance, which is at most 2.5 µM worth of biopolymer. From a biotechnological point-of-view, the cost of manufacturing dropped from 160€ to *ca.* 12€, which is a 13.3-fold reduction that could be re-invested into optimizing the solution to achieve the remaining 5% of post-thaw viability. However, given the error bars for each experiment, the difference in viability is negligible, which truly yields an interesting success with multiple substitutions.

It must be noted that Unisol-S3 contains HEPES as the buffer component. An additional experiment that is not shown in Figure IV.19 (Unisol-S2) showed that substituting the $H_2PO_4^-/HPO_4^{2-}$ buffer system by HEPES resulted in a 7% decrease in viability (see Section IV.7.1). Although these substitutions do not follow such a linear interpretation of facts, it is possible that using the $H_2PO_4^-/HPO_4^{2-}$ buffer system on Unisol-S3 could yield the highest cell viability for all Unisol-CV test conditions.

Bear in mind that combinatorial substitution is a qualitative approach. That is, its objective is constrained to narrowing down the combinations that provide the best practical result, which is a balance between post-thaw viability and number of substitutions. From a biomedical point-of-view, highest viability is the key factor because it provides the greatest probability of survival in the case of multicellular systems like whole organs. This is especially important in high-profile samples

that are very rare in quantity and must be preserved. From a biotechnological point-of-view, increasing number of substitutions results in (i) reduced production costs for a given formula and (ii) increased added-value to FucoPol as a bio-based product. Therefore, maintaining the original viability value while simplifying a formula is already a positive outcome. The clash of both interpretations requires a balance to be met between practical and economical aspects, depending on the field of application of this methodology.

In summary, FucoPol not only demonstrated to have multiple properties but it also proved to be multifunctional in a cryoprotective formula, evoking such properties to operate simultaneously. Although some constituents are essential, others can be compensated but this methodology is imbued with unpredictability that can only be fully elucidated through modelling of a molecular interaction network.

7. Ratio-based modelling: a method of analysis

A cryopreservation assay that is based on formula optimization yields a considerable amount of data to be interpreted, and often the researcher may find himself analyzing redundancies that could be overcome if target interactions between substances were mapped. In a way, it would ease the process of optimization if the amount of combinations between the constituents that yield greater viability could be narrowed down.

This section attempts to derive some main conclusions from a fully empirical dataset in the pursuit of a semi-empirical method of analysis. Figure IV.20 represents a proposed interpretation that could reinforce the foundations of a descriptive model for combinatorial substitution. Without neglecting the issue of ion confounding, behavioral patterns can still be identified and correlated with structural and functional characteristics, enabling the narrowing down of substance selection in high-throughput screenings.

There are four analytical checkpoints in which deducing a specific viability ratio can give information about the system tested. The screening was divided in two phases of experimental analysis. Phase 1 is mostly based on empirical trial and error and is the practical segment of the model: it concerns the contribution of the target constituent to be removed that the supplementation substance to be added to the formula (in this work, FucoPol). Phase 2 is mostly an analytical segment that relies on consequential interpretation of the results obtained: it attempts to define how both substances affect each other and the end result that is obtained from the substitution.

The schematic plot in Figure IV.20 summarizes the comparisons that can be performed based on how the experiment was carried out, depending if the formula was normothermic or hypothermic. In normothermic formulas, the original formula (C) was either supplemented with FucoPol (C^+), deprived from a target constituent (T) or both (T^+). As four post-thaw viabilities were obtained in this fashion, there are four relevant ratios to be calculated, so normothermic experiments have a 4-step interpretation. In hypothermic formulas, the removal of a constituent and addition of FucoPol was a simultaneous experiment, so its interpretation is restricted to step 4. All steps can be performed on whatever formula type, but the amount of experiments were reduced for the sake of time.

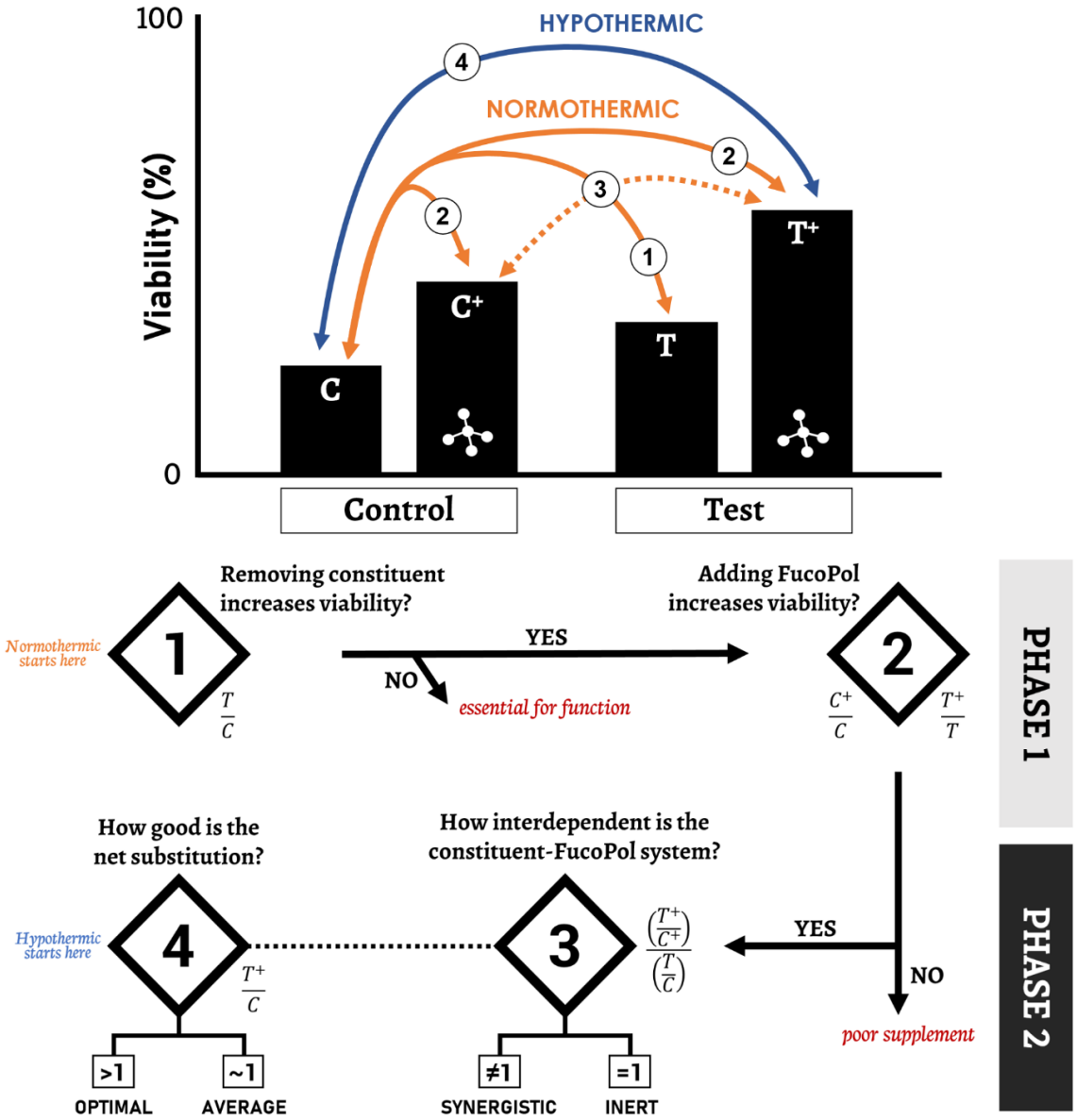


Figure IV.20. Theoretical bar graph (top) and corresponding flowchart (bottom) for the analysis of the contribution of FucoPol and other constituents for cell viability in the tested formulas. The step-by-step analysis is done by the numerical sequence displayed (1–4), in which specific ratios are calculated and give information on either the importance of the constituent or FucoPol for the final outcome. Given a control formula C, three conditions can be analyzed: upon addition of FucoPol (C^+), after removal of the target substance (T) or both (T^+). All four combinations were performed in normothermic formulas (orange) whilst the incremental substitution method applied to hypothermic formulas only yields the initial and final states (blue).

Relevant test conditions are subjected to analysis by following the numerical sequence of the flow chart in Figure IV.20. Each step contains a research question and a mathematical calculation from which a verdict can be based on marginal parameters.

1. **The constituent:** the first analysis concerns the targeted substance to be removed from the formula. A post-thaw viability ratio between its absence (T) and presence (C) will demonstrate how beneficial it was in cryopreserving the biological sample. The conclusion here is quite straightforward: a ratio higher than 1 indicates that viability increased, and

the constituent did not benefit the system. A ratio lower than 1 indicates that removing the constituent is harmful to the system, therefore essential for function.

2. **The supplement:** in this work, FucoPol was chosen supplement for implementation. Analyzing the ratios between solutions whose sole variable is its presence or absence yields its contribution to the performance of the formula: formula 2.1 reflects the ratio of control samples and formula 2.2 reflects the ratio of test samples. In fact, this comparison was consistently performed throughout the thesis, and FucoPol contributed to higher viabilities (ratio > 1). If a ratio of 1 or lower is observed, the supplement is considered either non-contributive or a poor option, respectively, therefore being discarded.

3. **Interdependencies:** the third step is a ratio of ratios. It compares a change in viability in the presence of FucoPol to a change of viability in its absence between control and test conditions. Essentially, formula 3 normalizes the activity of FucoPol to the absence of the target substance and can be expressed as:

$$\frac{(\frac{T_+}{C_+})}{(\frac{T}{C})} \quad or \quad \frac{(\frac{\mathrm{T}_+}{\mathrm{C}_+})}{\textbf{Formula 1}}$$

A ratio > 1 indicates that the performance of FucoPol is higher when the constituent is removed than when both substances are in solution. This means that their performances must be interdependent and reveal a case of non-ionic confounding. A ratio of 1 indicates that the performance of FucoPol is not affected by the presence or the absence of the constituent, so there is no polymer-constituent interaction.

It is assumed that the addition of FucoPol will result in a proportional ratio of change. Previous experiments demonstrated that the addition of FucoPol always had a noticeable effect on viability. Therefore, whether constituent removal is beneficial or not, FucoPol will always influence the final result. This postulates that a ratio < 1 is only possible if the addition of FucoPol is inhibiting the beneficial properties of some compounds already present (while a decrease in viability upon FucoPol addition was never observed, let this be a theoretical observation for general cases). However, because post-thaw viabilities only reflect the end-state of a system, it is impossible to differentiate which substance harmfully acts upon which. Therefore, an interdependency can only be a two-state system: when ratio = 1, there is no dependency between substances and their interaction is considered inert; when ratio ≠ 1, they influence each other, and the system is synergistic (either beneficial or harmful).

4. **Net quality:** lastly, this parameter reflects the overall adequacy of a given substitution to improving the viability of a living system. Given that substituent removal (step 1) and supplement addition (step 2) is a simultaneous process, the ratio carries a cumulative contribution, and formula 4 can be expressed as a multiplication of both stages:

$$\frac{\mathrm{T}_+}{\mathrm{C}} \quad or \quad \frac{(\text{Formula 2.2})}{(\frac{1}{\text{Formula 1}})} \quad or \quad \mathbf{F1 * F2.2}$$

If ratio > 1, it means both actions jointly contribute to a performance increase and the optimization step is considered optimal. If the ratio is *ca.* 1, then either step did not have a positive outcome and nullifies the beneficial effects of the other, so the substitution is considered average. In hypothermic formulas, a ratio < 1 can be obtained: in this case, the

supplement was not able to compensate for the harmful effects caused by the absence of the target constituent. Therefore, the substitution is considered ineffective for optimization purposes.

At the risk of overinterpretation, bear in mind that this analysis was designed to assess the system from a qualitative perspective. That is, the analysis has a practical nature: it tries to assess how good a formula can perform without elucidating the mechanism of action for each substance. In such depth, a new study on confounding must be done in order to clarify and discern each consequential effect in action. In spite of being superficial, it is hoped that this analysis lays ground on the study of molecular interactions between small and large molecules, with the goal of (i) shedding light on multifunctionality and (ii) characterizing how combinations can be performed to achieve maximum viability in cryoprotective formulas.

7.1. Testing the model in normo- and hypothermia

To validate the theoretical statements hypothesized, some specific substitutions were targeted to assess the adequacy of the model. Both the 4-step (for normothermic formulas) and 1-step approaches (for hypothermic formulas) are exercised to exemplify how the logical reasoning can sum up to a final verdict.

The screening of normothermic formula involved studying the importance of fetal bovine serum (FBS) in the DMEM growth medium. As briefed in Section I.5.1, earlier versions of cryoprotective formulas relied on animal-derived products to achieve adequate cryoprotection, which eventually led to the discovery of xenoimmunity. Fetal-derived serum has a low level of antibodies, which is advantageous in avoiding xeno-immune reactions, leading to undisturbed cell proliferation[189]. However, the biomedical community is becoming more and more intransigent to the use of bovine and calf sera due to ethical reasons, so there is an emergent need to substitute these products in complex cell media. Figure IV.21 shows the post-thaw viability of the four test conditions required to perform ratio-based modelling.

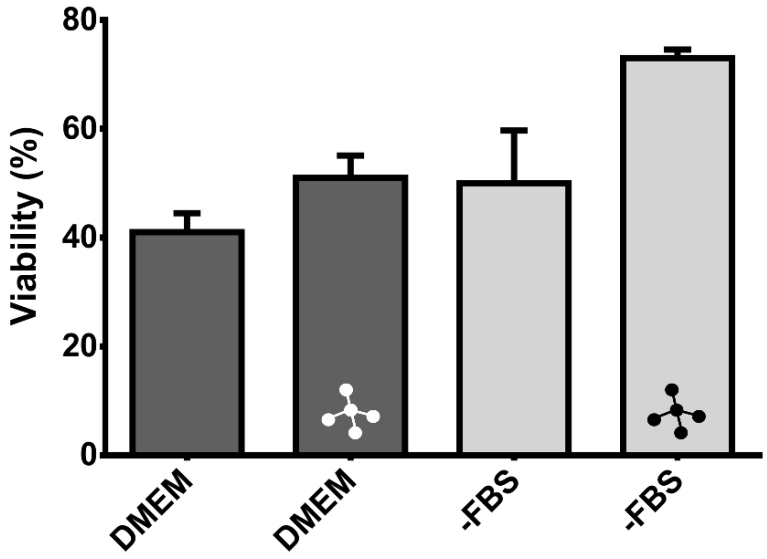


Figure IV.21. Comparison of the post-thaw viability obtained in culture medium with and without FBS. Notice that FucoPol can compensate for the absence of animal-derived products and even increases its performance in their absence.

DMEM is the control medium (C) to which FucoPol will be added (C^+). Simultaneously, the test condition is the removal of FBS from DMEM's composition (T), to which FucoPol will also be added (T^+). Once variable assignment is performed, formulas 1 to 4 are calculated based on the viabilities observed for each condition and individual verdicts are established, following the flowchart in Figure IV.20:

Variable	Condition	%	Ratio 1	Ratio 2.1	Ratio 2.2	Ratio 3	Ratio 4
C	+FBS	41	1.2	1.2	1.5	1.2	1.8
C^+	$+FBS^+$	51					
T	-FBS	50					
T^+	$-FBS^+$	73					
Verdicts			YES	YES		SYNERGISTIC	OPTIMAL

When we consider the contribution of FBS to the overall post-thaw viability of Vero cells (formula 1), we see that removing it from the formula's composition results in a 1.2-fold increase. On an independent experiment, formula 2.1 indicates that adding FucoPol in the presence of FBS results in a 1.2-fold increase, but a 1.5-fold increase in its absence. This difference promptly indicates that both substances might have some degree of confounding, as varying one changes the performance of the other. This is validated by assessing their interdependency (formula 3), which appears to present a synergistic effect ($R3 \neq 1$).

As expected, removing FBS enhances the performance of FucoPol. Looking at the overall net quality of the substitution, not only we infer that is was optimal ($R4 > 1$) but the ratio is higher than any other combination tested. This results from the cumulative contribution of both removing FBS and adding FucoPol yielding beneficial effects on cells. In summary, we can conclude that the presence of FBS is somehow decreasing the ability of cells to resist the hypothermic continuum and its absence can be compensated by adding FucoPol to the medium.

The following example pertains to a target substitution performed on hypothermic formulas, specifically Unisol-CV. The Unisol-S1 and Unisol-S2 test conditions correspond to identical formulas that vary on their pH regulator, the $H_2PO_4^-/HPO_4^{2-}$ buffer system and HEPES, respectively and the results are shown in Figure IV.22.

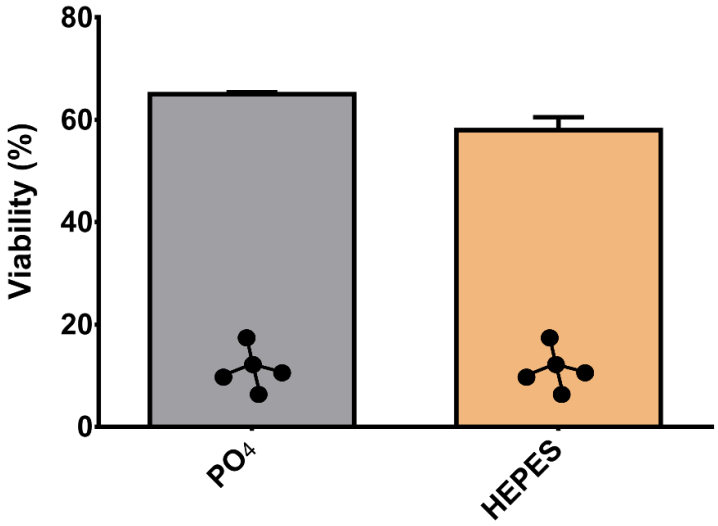


Figure IV.22. Comparison of the post-thaw viability obtained under different buffer compositions. In the presence of FucoPol, HEPES seems to yield a slightly higher recovery than a $H_2PO_4^-/HPO_4^{2-}$ conjugate buffer.

While FucoPol is present in both conditions, which would yield a C^+/T^+ comparison, bear in mind that the original Unisol-CV solution additionally contained gluconate and dextran that were removed in both of these conditions. To minimize the confounding effects that would influence the interpretation, the only variable in this substitution is therefore the buffer solution and FucoPol is assigned as a constant. In this case, the comparison is simpler because some variables are null, and only step 4 is assessed:

Variable	Condition	%	Ratio 1	Ratio 2.1	Ratio 2.2	Ratio 3	Ratio 4
C	PO_4	65	-	-	-	-	0.9
C^+	---	---					
T	----	---					
T^+	HEPES	58					
Verdicts			---	---		---	AVERAGE

Substituting $H_2PO_4^-/HPO_4^{2-}$ by the HEPES buffer system is not recommended, as viability decreased *ca*. 10%. It is arguable whether R4 = 0.9 fits into the R ~ 1 or the R < 1 parameter. From the point of view of considering that FucoPol is exerting a beneficial effect on the solution, a 10% decrease is not significantly harmful, so the substitution would be considered average. However, the substitution should be discarded as it does not contribute to optimization the Unisol formula.

These analytical observations are in agreement with the empirical results obtained and define the first version of an interactive model for cryoprotective formula optimization. While not descriptive mechanistically, this model still presents qualitative evidence that, when supplemented with knowledge of confounding, can reflect how a complex system behaves and how it will influence a biological system. This approach can benefit several fields of science, with emphasis on experimental biology and pharmacology and provides a tactic to systematically reduce the trade-off that exists between good performance and time-cost constraints, which severely deters the ability to develop novel solutions to challenges in emergent fields.

8. Preliminary results: looking further

This section compiles a subset of experiments performed with FucoPol and only reflect preliminary results. Besides the exploratory character of this work, these experiments try to complement even further some issues and minor details in polysaccharide science that can be found intriguing while studying their implementation in cryobiology and other areas.

Although these experiments are interpreted from a qualitative perspective, they allow for some conclusions to be taken and bridged with interesting future prospects that require answers in the current state of the art, thereby increasing knowledge in the minor features of these structures.

8.1. Minimal cryoprotective FucoPol concentration threshold

Thorough experiments with glycerol and DMSO over the years have shown a clear dependency between CPA concentration and cryoprotective activity. With the purpose of decreasing the market value of a FucoPol-containing formula, it was interesting to assess how much could reduce the amount of FucoPol used before the cryoprotective effect disappeared. In order to minimize the amount of FucoPol needed to observe significant cryoprotection, FucoPol solutions of 0.25, 0.13, 0.06 and 0.03% w/v were prepared and cell viability results are shown in Figure IV.23.

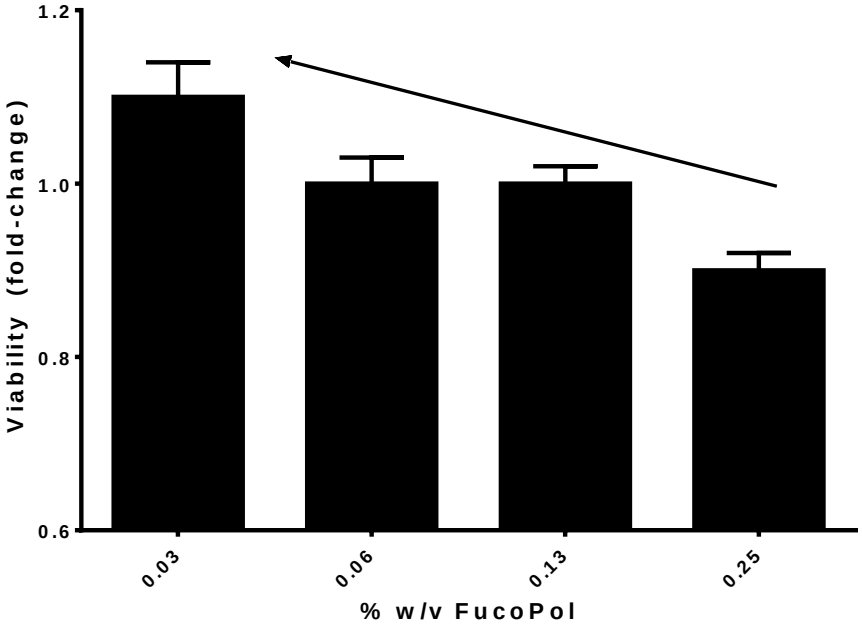


Figure IV.23. Post-thaw viability of decreasing concentrations of FucoPol to assess the minimal cryoprotective threshold. An increase was interestingly observed, probably due to a reduction of the osmotic stress cells suffer due to its extracellular presence.

Notice that concentrations below 0.4% w/v are not of interest to FucoPol in specific because this was the minimal concentration required to observe an antioxidant effect. For the sake of multifunctionality, only values above 0.4% w/v are considered of interest, as a balance between both properties must be met.

While it was expected that decreasing the concentration of FucoPol would result in decreasing viability, the opposite was observed. In fact, the working concentration 0.25% w/v FucoPol presented the worst viability results.

It has been clearly demonstrated that FucoPol can provide efficient cryoprotection when supplemented to the most diverse of formulas and is not cytotoxic to cells. Therefore, the most likely explanation for this phenomenon is that decreasing the amount of FucoPol is also reducing a concentration-dependent nefarious effect. Given the extracellular character of FucoPol, this might be any type of a solution effect, like osmotic stress, which hampers the ability of cells to maintain a correct cell volume and sustain cell volume variations upon addition and washing of the CPA.

8.2. Fucose content yields higher viability

The ability to prevent cryoinjury is largely correlated with molecular structure. However, biotechnology provides a way to manipulate biopolymer composition, which may influence structure altogether. However, the purpose of this experiment was to assess the contribution of fucose to the antifreeze potential of the fucose-rich FucoPol. Biopolymers produced from different sources with decreasing fucose content (A to D) were assayed and compared to FucoPol and the viability results are shown in Figure IV.24. Polymers A and B were produced by *Enterobacter* A47, much like FucoPol, but in different bioreactor conditions, which naturally yields different products. Polymer C was obtained from microalgae as part of an undergoing PhD project in the lab. Polymer D was produced by *Pseudomonas chlororaphis* and does not contains any fucose.

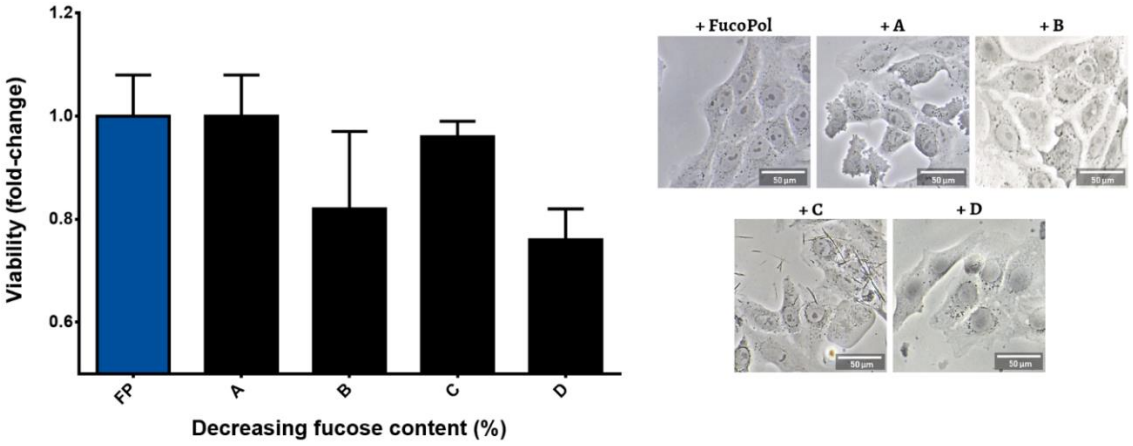


Figure IV.24. Post-thaw viability of different biopolymers of decreasing fucose concentration (from A to D). To the right is the morphological appearance of Vero cells under the presence of the five biopolymers tested. Fucose content seems to be proportional to cell viability. The viability for C was considered an outlying result attributed to the presence of harmful crystals, but shown nevertheless.

An increase in post-thaw viability was observed with increasing fucose content. It is uncertain if fucose has a specific chemical interaction with the crystalline matrix or the cell membrane that results in a conformational change of the polymer to better adapt to ice inhibition, but there is a clear tendency for increasing cell survival. However, cell morphology appears to be quite destabilized in the presence of all polymers except FucoPol, specifically in polymer D, which appears to cause cell agglomeration and swelling while disabling mitotic separation. Despite the similar viability of polymer A to FucoPol, a high number of apoptotic pockets are present on the cell surface.

Polymer C still had salt-derived crystalline fragments that could mechanically rupture the cell membrane – this is a possible explanation for its outlying viability value, as it is clearly not consistent with the morphology of Vero cells. In a condition of acute stress, cell metabolism reacts to unusual conditions by momentaneously increasing the metabolic rate[190], which could result in an overextrapolation of resazurin conversion.

Before the cryopreservation assay was performed, each polymer was sterilized for biological use, using the same standardized procedure for FucoPol that is detailed in Section IV.4.1. Polymers specifically produced by *Enterobacter* A47 have shown to balance fucose with its glucose content. Thus, when autoclaving low-fucose content polymers, it is possible that a high glucose content explains the observed partial caramelization of polymers B, C and D, which might have influenced their rheological properties and subsequent effect on viability. Additional optimization must be done when different biopolymers are assayed, as different properties require different thermal treatments.

8.3. Tackling the single weakness of DMSO

Since the first use of DMSO as a cryoprotectant that cytotoxicity has been a huge issue in the field of cryopreservation. In truth, DMSO is the least toxic of all CPAs tested in living systems[191] but the injury it causes is still lethal and capable of disrupting a whole organ if not washed from the system. In Section IV.4.3 it was shown that FucoPol is able to synergize with DMSO at the physical level and yield higher viability results, but we were unable to dissect if such an increase also stemmed from an attenuation of DMSO cytotoxicity at the chemical level.

To assess how FucoPol influenced the behavior of DMSO, Vero cells were exposed at 37ºC to DMSO for different periods of time, both in the presence and absence of FucoPol and the results are shown in Figure IV.25.

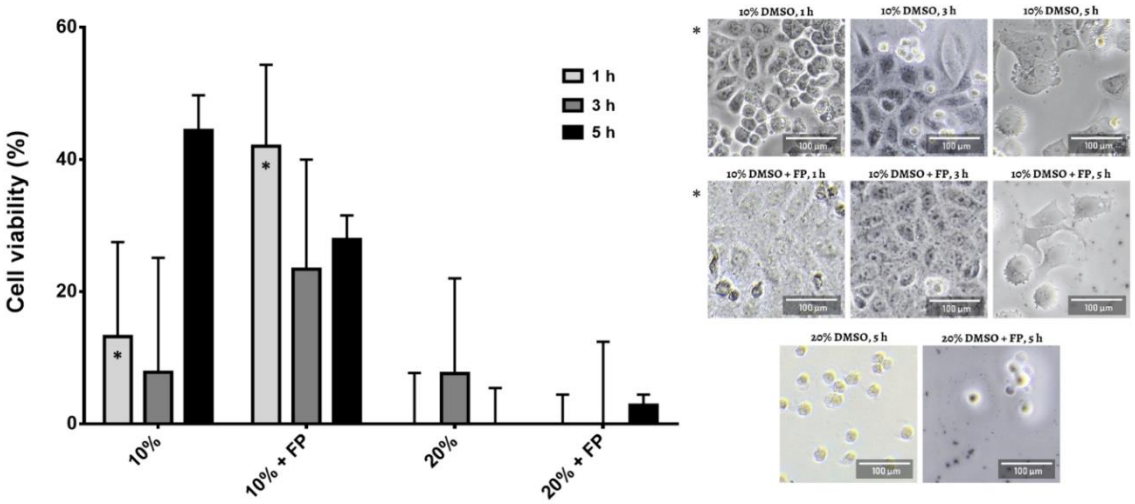


Figure IV.25. Viability at 37ºC after exposure to DMSO for different periods of time. To the right is the morphological appearance of Vero cells after exposure to the different mixtures. A less acute decrease in viability occurs when FucoPol is mixed with 10% DMSO. The standard conditions to which a normal cell line is usually exposed in freeze-thaw procedures is represented by an asterisk (*). In the 20% DMSO photographs (bottom-right), the presence and absence of FucoPol can be clearly visualized as black dot particles.

Cells in the presence of any concentration of DMSO are generally left at physiological temperature for the shortest amount of time possible before freezing. However, extreme exposure times were tested to see if FucoPol could have a significant influence in viability.

First, the addition of FucoPol to 10% DMSO seems to maintain a higher cell viability overall. As time lapses, cells in the presence of FucoPol seem to not suffer such an acute toxicity effect from the presence of the CPA. A concentration of 20% is just too lethal for cells or FucoPol to be able to counteract its effect and was an expected outcome.

Cell morphology was also assessed for every test condition and is presented to the right in Figure IV.25. After 1 h in 10% DMSO, cells detach and become spherical, a clear sign of cell death. After 3h, a lot of birefringent cells are observed, validating the detachment, but some started to re-adhere. After 5h, there are adhered cells but very deformed and surrounded by apoptotic pockets. When FucoPol was added, regular morphology is observed past 1 h of exposure, which is a good indication of an attenuation effect in practical terms. After 3 h, some birefringent cells are present but overall Vero cells are proliferating with normal morphology. However, 5 h of exposure lead once again to an apoptotic and swollen aspect.

At 20% DMSO, either in the absence of presence of FucoPol, a similar cascade was observed. An initial of 1 h resulted in some dead cells and the amount adherent healthy cells decreased over time. However, after 5 h there was a total inability to proliferate, even in a deformed state.

It is important to understand that before the 5-hour mark is reached, a washing procedure would already have been implemented and cells would not be exposed to DMSO for even longer than 1 h. In cryogenic storage, DMSO toxicity is not a problem because diffusion rates are incredible small at -80ºC, but perfusion procedures are when cells are at the most risk. Another aspect is that

although a time lapse of 1, 3 and 5 h is represented, what is observed might not correspond to a chronological event cascade, as each time point was an independent experiment.

In summary, a 3.2-fold increase in cell viability occurs when FucoPol is added to a 10% DMSO-containing medium. This is visualized when comparing bars that are marked with an asterisk (*) and they represent realistic conditions (concentration, time) to which a normal cell line would be exposed in a freeze-thaw procedure. It is already known that DMSO permeabilizes the cell membrane causing an unspecific molecular influx, as mentioned in Subsection I.4.2.2. However, this might not be a CTN effect per say, but a counteracting osmotic regulation by FucoPol, as there is no chemically interactive evidence that an intrinsic cytotoxicity reduction is occurring. This aspect is further detailed in Chapter V.3 for convenience.

8.4. Implementation of FucoPol in CryoStor™

Contrary to normothermic solutions like DMEM or hypothermic like Euro-Collins, Cryostor™ CS5 is a **cryogenic** solution optimized for the long-term storage of stem cells from -80 to -196ºC. It is a serum-free, animal component-free medium with 5% DMSO and it can be equally applied to tissue preservation as well. All major cryopreservation assays at -80ºC targeted the substitution of hypothermic formulas, designed for high subzero conditions (0-8ºC) which is counterintuitive. However, as previously mentioned, the goal of a cryoprotective formula is to guarantee that cells have the necessary resources to sustain the injuries caused by the hypothermic continuum. If a FucoPol-loaded hypothermic formula is efficient in a cryogenic environment, it is safe to assume that those beneficial effects are translational to cryogenic formulas.

With the intent of assessing if FucoPol could enhance the performance of a fully developed and marketed formula, a simple addition of 0.25% w/v FucoPol was done to CryoStor™ and the results are shown in Figure IV.26.

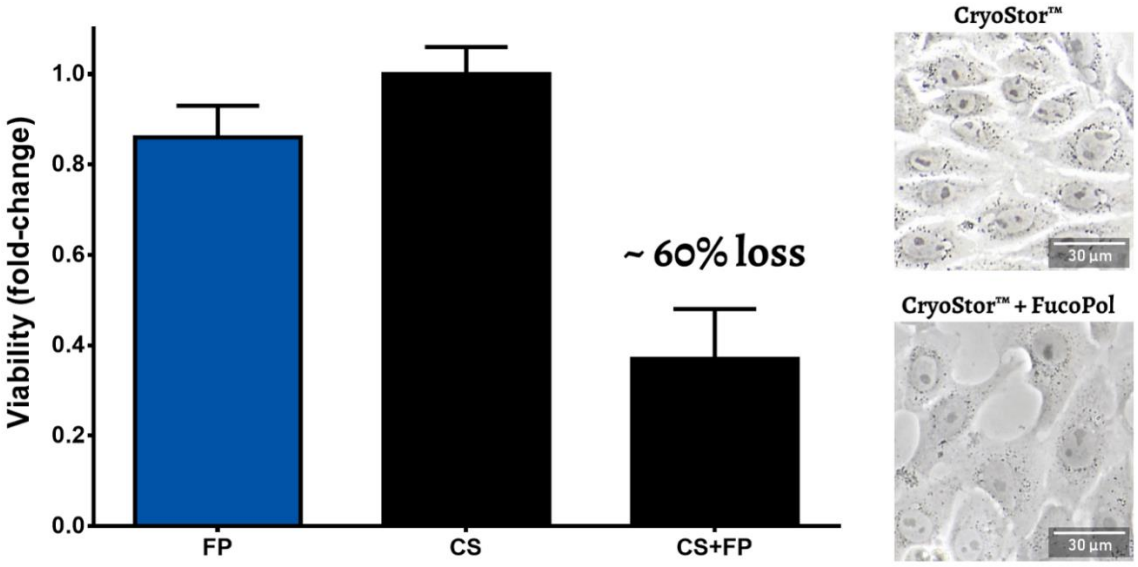


Figure IV.26. Post-thaw viability of Vero cells exposed to the original and FucoPol-loaded CryoStor™. A major loss of viability is observed when FucoPol is added to CryoStor™ but metabolic results seem to be incongruent with cell number and morphology (right). The blue bar represents the DMEM+FP solution for comparison.

Despite the positive results consistently observed throughout this work, mixing FucoPol with CryoStor™ resulted in a major decrease in viability. However, cell death is not apparent given normal cell morphology. In both cases, some apoptotic pockets formed in similar amounts, but cells did not produce agglomerates, swelling or stretching variations.

One possible explanation is a delayed resazurin diffusion that results from an increased medium viscosity, causing less molecules to permeate the cell membrane in a given time window. As such, a lower resazurin conversion would be masked as a lower cell viability in the case of CS+FP.

A second possibility, although less probable, is a conflictuous primary interaction. The more specific cryoprotective solutions are to a given cell line, tissue histology or freeze-thaw protocol, the more complex they can be. STEMCELL™ Technologies does not have full disclosure on the composition of their products, so it is uncertain what constituent could be antagonistic to FucoPol.

8.5. Shining light on cosmetic applications

It was briefly mentioned in Section IV.4.1 that UV irradiation was unable to sterilize a contaminated FucoPol sample and it was postulated that due to its characteristic opaqueness, FucoPol molecules present in the superficial layer of the solution could absorb all incident radiation, effectively protecting microorganisms in deeper layers.

To assess the photoabsorptive properties of FucoPol, a UV-VIS spectrum of a 0.25% w/v FucoPol sample was collected. Also, Vero cells were seeded to form an epithelial layer that mimicked the basic structure of skin and irradiated with UV light in the presence of FucoPol. The viability results obtained are shown in Figure IV.27, along with the associated experimental design.

The UV-VIS scan performed to a sterile FucoPol solution showed saturated absorbance values at 300 nm and lower, which is in the near ultraviolet region. This means that for every photon that is irradiated, 10,000 photons are most likely retained in the outer edges of the solution's volume.

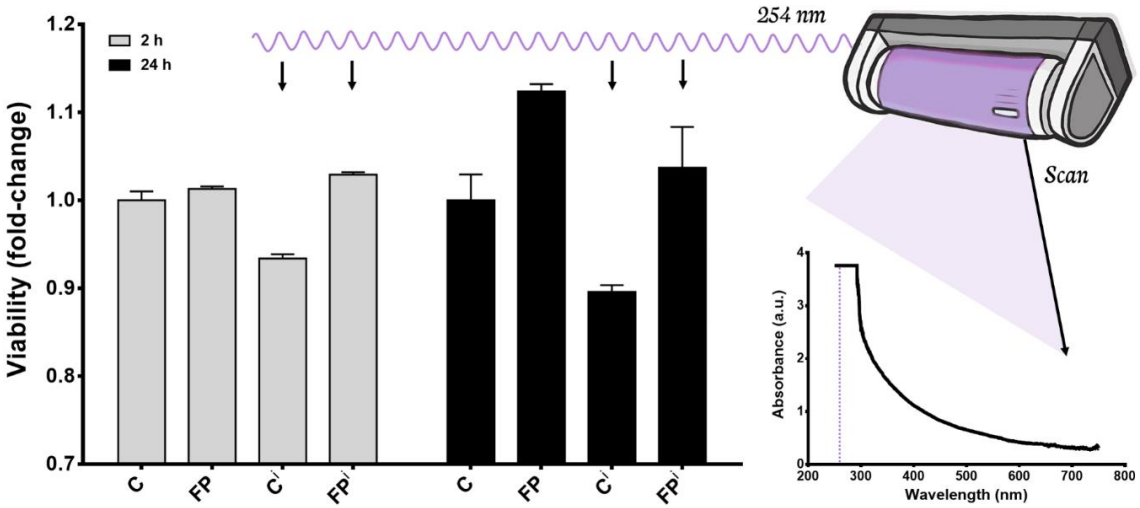


Figure IV.27. Experimental design to assess the photoabsorptive properties of FucoPol in acellular and *in vitro* environments. A scan spanning the 250–750 wavelength range showed transmittance values lower than 0.01% below 300 nm. After 30 min of irradiation at 254 nm, irradiated cells (represented by the i superscript) maintained a higher viability when FucoPol was present. The same tendency was observed after 2 and 24 h of incubation prior to viability assessment.

When Vero cells were irradiated with the 254 nm UV beam of a laminar flow chamber – used for sterilizing the environment – for 30 min, 10% more viable cells were observed in the presence of FucoPol after 2 h of the exposure period. To assess if the exposure to UV irradiation would result in delayed cell death, cell viability was assessed after 24 h of the experiment, but similar results were obtained, observing a 1.16-fold change.

DMEM was used as the control medium and it presents an orange coloring. It could happen that DMEM would equally absorb UV radiation, as observed with FucoPol, but this was not assessed. However, a practical point-of-view dictates that if a cell layer is irradiated with germicide UV radiation in the presence of FucoPol and can avoid substantial damage while in DMEM it cannot, then the color of the solution might not be a defining parameter of radiation protection in this case.

Although irradiation intensity was not controlled, it is more aggressive in the selected time window than solar exposure, as it is meant to be germicide. These results are promising for cosmetic applications, mostly in the industry of sunscreens. The photoabsorptive properties of FucoPol could be further enhanced with additional pre-treatments of the biopolymer: sunscreens usually contain heavy metals that act as UV filters that are thought to be toxic. Heavy metal inclusion in FucoPol microspheres could potentially reduce its epithelial diffusion and toxicity whilst still providing solar protection.

V. CONCLUSIONS

1. Take-home message

FucoPol is a fucose-rich polysaccharide that was produced by *Enterobacter* A47 in a bioreactor cultivation with glycerol as the carbon source. The polymer was recovered from the broth and purified by dia/ultrafiltration using a 100 kDa cut-off membrane.

As FucoPol was envisioned to be a constituent of cryoprotective fluids, its physicochemical properties were assessed to check for significant variations. FucoPol at 0.25% w/v was used as the working concentration for all subsequent experiments and presented a dynamic viscosity value of 1.46 mPa·s at room-temperature and a pH of 6.1. When added to BSA or Euro-Collins solutions, a decrease in viscosity was observed. Given that FucoPol has a shear-thinning behavior, this observation is consistent with its application in cryoprotective formulas, as the target vasculature is always under a constant pressure, either from heart pumping or an artificial device. It also did not show to undergo any conflict with essential constituents, proving to be a beneficial addition.

The ability of FucoPol to provide cryoprotection is not related to achieving a state of vitrification but a similar behavior to an antifreeze protein. In flash freezing assays, no concentration of FucoPol was able to avoid a very fast ice growth. Instead, FucoPol acts by facilitating an earlier crystallization event, resulting in less crystal growth, both in number and size. Also, it acts by destabilizing the crystalline network of any ice formed, which results in a lower melting point. This effect seems to be dose-dependent and thermal hysteresis increases in the same fashion as a type I AFP by a non-colligative mechanism of action. When mixed with either BSA or AFP, it does not reach optimal antifreeze activities, but it does not decrease its activity in their presence. This is beneficial in living systems, as a great diversity of molecules could negatively impact its ability to provide cryoprotection.

Before implementation in cryopreservation assays, FucoPol was sterilized by dry autoclaving, a method that preserved both its aspect and dynamic viscosity, the key indicators of structure and function. FucoPol was not cytotoxic to any cell line studied and showed to maintain cell morphology both before and after a freeze-thaw procedure. When added to normothermic formulas, all had an increased performance on cryopreserving cells, proving once more that FucoPol can adapt and function in different ionic compositions.

Given the hypothermic continuum cells must undergo in a freeze-thaw procedure, hypothermic formulas are more complex as different functionalities have to be addressed. However, complexity is closely associated to cost of production, and these formulas are used very often, in large quantities, just to be washed again to avoid any cytotoxicity at physiological temperature. In order to reduce the expensive burden on its usage, FucoPol was implemented in different hypothermic formulas by testing the removal of different constituents.

It was postulated that FucoPol could act as a colloid stabilizer and an osmotic regulator given its inherent properties to tackle physical damage. However, mitochondrial-induced cell death is a chemical event usually counteracted by usage of antioxidants, some of them being expensive redox enzymes. FucoPol demonstrated to have a dose-dependent antioxidant activity by effectively reducing Fe^{3+} species in solution. When results were extrapolated to the working concentration, not only FucoPol showed it could provide protection similar to vitamin C, it also protected Vero

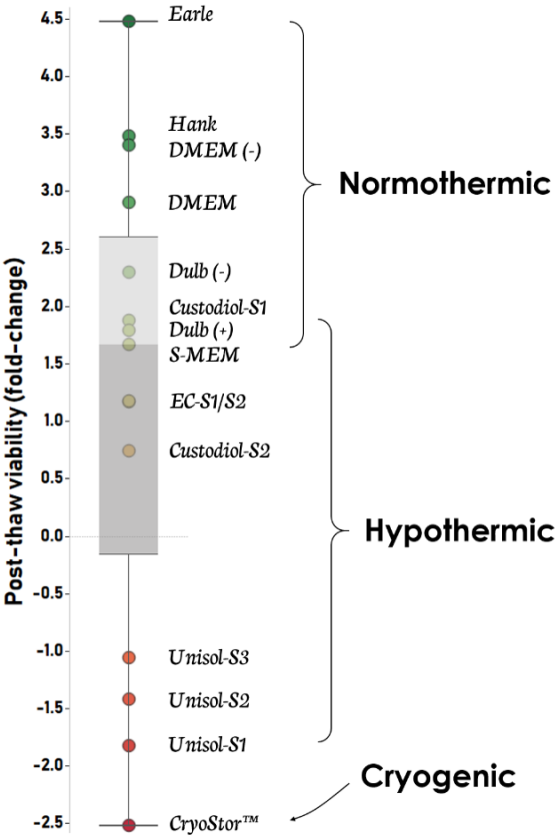


Figure V.1. Statistical box-plot of the 15 tested conditions reflecting a 67% viability success rate. The data encompasses 1 cryogenic, 7 hypothermic and 7 normothermic formulas which were subjected to the presence of 0.25% w/v FucoPol. Success cases were considered to have a fold-change > 1.0 and a median of 1.663 was obtained for this dataset. The values plotted are a ratio between the viability at 37ºC and after freezing for a given solution, which provides a realistic measure of a formula's adequacy for physiological implementation. Notice that normothermic formulas appear to be surprisingly better than the ones tailored for sub-zero temperatures. Whiskers represent marginal values of 4.475 and -2.520. Hinges represent data within the 1.5 IQR (inter-quartile range) and have values of 2.600 and -0.160.

cells against H_2O_2-induced oxidative damage by efficiently negating the effects of ROS. Cells did not perish as much in the presence of FucoPol but also recovered faster from the acute stress.

Once proved that FucoPol could have different properties equally applicable to hypothermic formulas, a set of substitutions was performed, in which the solutions were incrementally stripped of their original constituents to assess the ability of FucoPol to compensate for such deficits. Whilst some compositions are essentially dependent on their impermeant solutes (case 1), the absence of some constituents can be compensated (case 2) to a point that several substances can be removed and the performance maintained just by FucoPol addition (case 3). Figure V.1 is a statistical box-plot that gives a realistic summary on formula implementation results. Of all 15 conditions tested, 10 presented better results in the presence of FucoPol than their original counterparts, yielding a 66% success that reflects a randomized set of substitutions. Overall, a median viability increase of 1.663-fold was obtained, which indicates that FucoPol can potentially benefit most formulas currently developed.

Hypothermic and cryogenic formulas are very complex and less tolerant to the presence of other substances, which is why despite small increases in performance they are below median. An upwards tendency (to positive values) indicates that post-thaw viability is increasing with increasing substitutions. However, the positioning on the positive or negative region of the graph reflects the ratio between post-thaw and 37ºC viabilities. For example, Unisol-CV saw a gradual post-thaw increase from S1 to S3 but viabilities at 37ºC were below that of the control solution (Unisol-C). Despite an increased cryoprotective effect, cells exposed to Unisol at 37ºC do not adapt efficiently to their nutritive source, reflecting the unoptimized state of this formula.

While these results are very promising in developing novel formulations, the optimization method is still purely empirical and lacks understanding on what trade-offs are done for a high quality final result. Besides the inconvenient issue of ion confounding, removing a substance whose function we do not fully understand can provide greater viability in short term but be detrimental to biological survival in long term from unknown circumstances. Therefore, a method of analyzing these substitutions is proposed in this work. From a brief application of the model in experimental

data not used to develop its logic, two observations were made. It was found that FBS, a constituent of growth media usually frowned upon due to its animal origins, actually had a negative contribution to post-thaw viability, and its presence was hindering the ability of FucoPol to successfully cryopreserve cells. Also, HEPES, which was commonly adopted for some formulations instead of the $H_2PO_4^-/HPO_4^{2-}$ conjugate buffer, hereby showed a worse contribution to Unisol formulations based on FucoPol.

While this method does not even begin to depict the full extent of molecular dynamics involved, the reasoning presented here hopes to encourage the development of predictive models in which substitution experiments can be based on. This would enable to reduce expensive and randomized high-throughput screenings to a narrow selection of substances better equipped to yield greater viability for any biological sample, significantly reducing their cost and lifting the financial burden off of hospitals and preservation centers, to better re-allocate their funds to different parts of the whole process.

In summary, three key aspects should be emphasized:

1. **Shifting paradigms:** research on formula optimization has been working around different substances and techniques rather than employing different fields that are able to produce different resources. Organic chemistry has been deeply involved in the synthesis of ice blockers but solely envisions the antifreeze property. The field of biotechnology provides a myriad of unearthed resources that are fully tailorable to their application and, as FucoPol, can be multifunctional. The ability to produce biopolymers with specific molecular structure and composition is a powerful asset in any field of science that urges for innovative solutions to resilient problems.

2. **Simplification:** a formula that contains less constituents tends to be less expensive. However, there are two more factors that are a consequence of simplifying formula compositions. On one hand, the immense diversity of cryoprotective fluids in the market can be dissipated: as formulas are composed of even more tailorable and multifunctional substances, specificity decreases because the requisites of different organs and methodologies can eventually be fulfilled by dozens instead of hundreds of designated formulas. On the other hand, substituting multiple constituents by FucoPol actually decreases the amount of variables that must be considered when developing predictive models. Different biological samples react differently to a given composition for reasons that so far have remained unknown and unpredictable, but most likely are related to a complexity degree that disables a differential diagnosis. Thus, eliminating harmful constituents once thought to provide essential function can reduce cell-related variability and allow to pinpoint key factors in cryodynamics. It then becomes clear that simplification breeds efficient standardization.

3. **Patterning:** last but not least, no simple method can be trustworthy of implementation if its logic is not totally understood. The field of preservation, just like cancer research, has long urged for the identification of patterns that allow us to build knowledge on how to best solve the current challenges. Developing mathematical models with close connection to statistics can help in narrowing down the best toolkit to achieve efficient preservation in a predictable way. Not only simulating outcomes can provide new information but also question implicit truths that, when once thought to be unshakable, now may prove to be nothing but suggestive at this stage.

2. Valuable optimizations

Some key changes can be performed when reproducing the presented work to achieve better results. Developing cryoprotective formulas is a complex study that involves several variables, and some were ignored in the expectation that they would have a negligible influence on the final result.

Osmolarity is a key parameter in ensuring cell survival after a freeze-thaw cycle, as the cell suffers severe volume changes both due to phase transitions and manual addition and removal of the solution. Given that all formulas were already optimized to have an osmolarity ranging from 280 to 350 mOsm, an osmometer was not used to assess if there was any variation upon the addition of FucoPol. In solutions where some constituents were eliminated, particularly impermeant substances, it was considered that FucoPol could eventually compensate the imbalance in final osmolarity and the cell would not undergo lethal osmotic effects. However, the results do not reflect if this had any influence, but it is important to assess when trying to implement a specific composition in the market.

The cell viability assays were done in 96-well microplates instead of 2 mL vials. Also, the styrofoam recipient was used as an alternative to Mr. Frosty (a recipient optimized for storage of biological samples), reported to ensure a cooling rate of 1ºC/min[192]. Given that this work heavily focused on formula optimization, technical details were slightly overlooked, and the viabilities obtained were most likely not maximal. There are three concepts worth mentioning:

- A high-throughput screening assay in a microplate involves using µL volumes instead of mL, which can result in reduced viability due to the more damaging network solidification instead of progressive solidification[193]. Is it recommended to narrow the best test conditions derived from a large screening and try to reproduce the same results in cryovials.

- The microplate itself does not a have radial symmetry of thermal distribution, which means inner wells will cool slower than outer wells, resulting in cooling rate variations that influence the results. If the disposition of replicate samples is incorrect, the results can be biased to a point that a comparison to other test conditions is unfaithful.

- It has been shown that all storage devices have slightly deviated cooling rates from what is reported by the manufacturers (CRYO2018, *unpublished*). Given that the styrofoam recipient was a rough adaptation to microplate storage and still presented a cooling rate of 1ºC/min when assessed with a thermometer, it is possible that the realistic deviation is even higher.

Relating to hypothermic formula substitution, a 4th composition was tested, Viaspan^{TM}. The substitutions tested were valuable proofs-of-concept that FucoPol could potentially substitute starch-based solutions as a colloid agent. However, all Viaspan-based solutions presented pH of 12, even in the presence of buffer conjugates, which resulted in massive cell death. While all other hypothermic formulas maintained their pH, Viaspan yielded unfortunate results inadequate for interpretation.

Finally, the usage of resazurin is a cost-efficient but troublesome method of assessing cell viability. Given that color acquisition is fully dependent on the metabolic work of cells, it can yield biased results if cells experience variable metabolic rates, which can derive from different nutrient

availability, different cell lines, exposure to xenosubstances or even their ability to recover from the freezing-related metabolic arrest. Cell viability based on DNA count, cell count or LIVE/DEAD™ assays that involve double fluorescent labelling could be more reliable methods in assessing metabolism-uncoupled cell viability and morphology changes at the subcellular level[194].

Post-thaw apoptotic detection is also important to ensure cells do not become dysfunctional after storage, and some assays involve annexin V-FITC labelling to check for cytosolic levels of mitochondrial phosphatidylserine[195], caspase-3 content monitoring with a mitochondrial (red) and cytosolic (green) MitoTracker fluorescent marker[196]. Band densitometry of the recovered cell pellet could also ascertain the presence of necrotic, apoptotic or healthy cells by the morphology of their DNA, as it could be randomly cut, fragmented in 120 base pair nucleotides or in the native state, respectively[197].

3. Future prospects

There are some challenges in the field of cryobiology that still remain to be addressed, whilst other fields of science can benefit from the usage of FucoPol. The preliminary results section of this thesis aimed to further understand how this specific biopolymer would behave for different applications as a means to increase its added value that stems from bioproduction.

As mentioned before, fucose is a high-profile monomer that is rare in nature. However, it is present in human breast milk, which has an optimal nutritive value with large contents of lactose, fucose and human milk oligosaccharides (HMOs), especially fucosyllactose, playing an important role in the immune system[198]. Therefore, the ability to produce fucose-containing saccharides is of high interest for the food industry. Fucose seems once more to be related with the cryoprotective ability of biopolymers, as it was observed in Section IV.8.2 that a higher fucose content resulted in higher cell viability. Whether fucose has a special structural interaction with ice crystals or is acting as a nutritive source, the mechanism still remains unknown, though results are still positive.

The photoabsorptive properties of FucoPol also seems to be a most promising prospect in the field of cosmetic applications. Not only the polysaccharide is able to absorb damaging UV radiation but also protects cell from radiative damage. Suncreens usually contain titanium dioxide or oxidized zinc that act as reflective substances. Although it is uncertain if these heavy metals could have harmful effects due to epidermal contact[199], the population becomes increasingly wary, which does not bode well with cosmetic companies. Some lotions are considered chemical sunscreens due to the use of oxybenzone instead of titanium and zinc.

However, research has shown relationships between oxybenzone and increased risk of melanoma and endocrine disruption as a mimic of estrogen[200]. Once more, the data is far from conclusive as many parameters can influence the outcome, but the implementation of FucoPol as a proven biocompatible substance could prove to be very valuable in overcoming these obstacles, either by formulating sunscreens with both substances or FucoPol alone. The ability of tailoring FucoPol to adopt different structural conformations (emulsions, encapsulative microparticles, and more) would further increase its potential to the field.

Finally, some concerns on cryobiology can be addressed given the preliminary results obtained:

- The first one is the unsolvable cytotoxicity challenge still faced by the use of DMSO. On one hand, some preliminary results showed that the presence of FucoPol could significantly attenuate the cytotoxic effects of DMSO at 37ºC. On the other hand, decreasing concentrations of FucoPol could be reducing a harmful osmotic effect, resulting in a viability increase. As such, two different events have an equal possibility of occurrence. Upon addition of 10% DMSO to the cryoprotective formula and, subsequently, to cells, the cell water volume will tend towards 111% of the initial volume. Upon thawing, removal of the medium due to its potential toxicity is usually done by diluting the 10% DMSO to at least 2.5%. In this case, cells will tend towards a water volume of 2.6 times the isotonic water volume, which might bring the cells close to their hypotonic limit. Putting a non-penetrating solute into the medium (like FucoPol) will reduce both the first 111% and the second 260% volume extremes, thus protecting the cells against the osmotic effects of DMSO. That would masquerade as a reduction in actual DMSO toxicity but would not be true toxicity reduction.

  Therefore, it is important to assess if FucoPol is having an anti-osmotic or an anti-toxic effect. A possible experiment to differentiate these mechanisms would be to compare FucoPol to an osmotic buffer like sucrose or mannitol in equal molar concentrations and check for any viability increase to the same extent of the polysaccharide.

- The second challenge relates to organ perfusion. FucoPol is negatively charged at physiological conditions and has a very high molecular weight, rendering it non-penetrating to cells. In organs, FucoPol can only access cells that are close to the vascular system but would not be near the innermost cells, which would result in variable cryoprotection. As a comparison, serum albumin (66 kDa) does not leak out of the capillaries very quickly. The charge might reduce leakage or result in some interaction with the capillary wall that could be negative, but experimental determination is still required to conclude. The main question to address here is “how big is big”, which stands in need for testing capillary diffusion models and assessing the permeability of FucoPol (and the whole formula itself) through interstitial spaces.

It was greatly stressed out throughout this thesis how progress in the field of preservation can benefit multiple disciplines in the field of biomedicine. Novel solutions based on biopolymers can provide an acquired economical advantage that is a big determinant of scientific evolution, further eliciting more funding as the current challenges become more elucidated and with greater hope of resolution.

The most distinct connection to be made is with tissue engineering. Idealistically, developing a FucoPol-based cryoprotective formulation that can efficiently preserve artificial organs produced is an assurance that mass production can be done without significant money loss. The resources produced can be readily used or stored, depending on what is convenient, and both fields can greatly advance as the economic barrier gradually dissipates. Both these factors allied with an efficient modelling strategy can remove any empirical drawbacks from the writing of research grants, and preservation science slowly transforms from odd-based discoveries to simulating the best outcome without wasting resources.

REFERENCES

1. Giwa, S. *et al*. The promise of organ and tissue preservation to transform medicine. *Nat. Biotechnol.* **35,** 530–542 (2017).

2. Cooper, D. K. *et al*. Report of the Xenotransplantation Advisory Committee of the International Society for Heart and Lung Transplantation: the present status of xenotransplantation and its potential role in the treatment of end-stage cardiac and pulmonary diseases. *J. Heart Lung Transplant.* **19,** 1125–65 (2000).

3. Population of Africa (2018) - Worldometers. Available at: http://www.worldometers.info/world-population/africa-population/. (Accessed: 23rd September 2018)

4. Liu, D. & Pan, F. Advances in cryopreservation of organs. *J. Huazhong Univ. Sci. Technol. - Med. Sci.* **36,** 153–161 (2016).

5. Khademhosseini, A. & Langer, R. A decade of progress in tissue engineering. *Nat. Protoc.* **11,** 1775–1781 (2016).

6. Sharp, P. A. Meeting global challenges: Discovery and innovation through convergence. *Science (80-.).* **346,** 1468–1471 (2014).

7. Larson, D. J. *et al*. Wood frog adaptations to overwintering in Alaska: new limits to freezing tolerance. *J. Exp. Biol.* **217,** 2193–2200 (2014).

8. Wszola, M. *et al*. One-year results of a prospective, randomized trial comparing two machine perfusion devices used for kidney preservation. *Transpl. Int.* **26,** 1088–1096 (2013).

9. Lai, J. C. *et al*. Frailty Predicts Waitlist Mortality in Liver Transplant Candidates. *Am. J. Transplant.* **14,** 1870–1879 (2014).

10. Morrissey, P. E., Flynn, M. L. & Lin, S. Medication noncompliance and its implications in transplant recipients. *Drugs* **67,** 1463–81 (2007).

11. Ravikumar, R., Leuvenink, H. & Friend, P. J. Normothermic liver preservation: a new paradigm? *Transpl. Int.* **28,** 690–699 (2015).

12. Bruinsma, B. G. *et al*. Subnormothermic Machine Perfusion for *Ex Vivo* Preservation and Recovery of the Human Liver for Transplantation. *Am. J. Transplant.* **14,** 1400–1409 (2014).

13. Fahy, G. M. *et al*. Cryopreservation of organs by vitrification: perspectives and recent advances. *Cryobiology* **48,** 157–178 (2004).

14. Campbell, B. K. *et al*. Restoration of ovarian function and natural fertility following the cryopreservation and autotransplantation of whole adult sheep ovaries. *Hum. Reprod.* **29,** 1749–1763 (2014).

15. Storey, K. B. & Storey, J. M. Tribute to P. L. Lutz: putting life on `pause' - molecular regulation of hypometabolism. *J. Exp. Biol.* **210,** 1700–1714 (2007).

16. Acceptable Ischemic Times | Nebraska Organ Recovery - Organ Donation in Nebraska. Available at: http://www.nedonation.org/donation-guide/organ/acceptable-ischemic-times. (Accessed: 23rd September 2018)

17. Chen, K. H., Boettiger, A. N., Moffitt, J. R., Wang, S. & Zhuang, X. Spatially resolved, highly multiplexed RNA profiling in single cells. *Science (80-.).* **348,** aaa6090-aaa6090 (2015).

18. Huang, Y., Williams, J. C. & Johnson, S. M. Brain slice on a chip: opportunities and challenges of applying microfluidic technology to intact tissues. *Lab Chip* **12,** 2103 (2012).

19. Manuchehrabadi, N. *et al*. Improved tissue cryopreservation using inductive heating of magnetic nanoparticles. *Sci. Transl. Med.* **9,** eaah4586 (2017).

20. Bischof, J. Nanowarming: A new concept in tissue and organ preservation. *Cryobiology* **71,** 167 (2015).

21. Boyden, E. S. A history of optogenetics: the development of tools for controlling brain circuits with light. *F1000 Biol. Rep.* **3,** 11 (2011).

22. Mali, P. *et al.* RNA-Guided Human Genome Engineering via Cas9. *Science (80-.).* **339,** 823–826 (2013).

23. Samadikuchaksaraei, A. Scientific and industrial status of tissue engineering. *J. Biotechnol.* **6,** 2897–2909 (2007).

24. On-Demand Cell and Tissue Biologics for Mass Casualty Response | SBIR.gov. Available at: https://www.sbir.gov/sbirsearch/detail/870637. (Accessed: 23rd September 2018)

25. Donnez, J. *et al.* Ovarian tissue cryopreservation and transplantation: a review. *Hum. Reprod. Update* **12,** 519–535 (2006).

26. HHS Boosts Stockpile of Products to Treat Acute Radiation Syndrome | EMS World. Available at: https://www.emsworld.com/news/11178384/hhs-boosts-stockpile-products-treat-acute-radiation-syndrome. (Accessed: 23rd September 2018)

27. Knebel, A. R. *et al.* Allocation of Scarce Resources After a Nuclear Detonation: Setting the Context. *Disaster Med. Public Health Prep.* **5,** S20–S31 (2011).

28. Shu, Z., Heimfeld, S. & Gao, D. Hematopoietic SCT with cryopreserved grafts: adverse reactions after transplantation and cryoprotectant removal before infusion. *Bone Marrow Transplant.* **49,** 469–476 (2014).

29. Ben-Bassat, H. Performance and safety of skin allografts. *Clin. Dermatol.* **23,** 365–75 (2005).

30. Ziegler-Graham, K., MacKenzie, E. J., Ephraim, P. L., Travison, T. G. & Brookmeyer, R. Estimating the Prevalence of Limb Loss in the United States: 2005 to 2050. *Arch. Phys. Med. Rehabil.* **89,** 422–429 (2008).

31. Dubernard, J.-M. *et al.* Human hand allograft: report on first 6 months. *Lancet* **353,** 1315–1320 (1999).

32. Dubernard, J. M. *et al.* Functional results of the first human double-hand transplantation. *Ann. Surg.* **238,** 128–36 (2003).

33. Devauchelle, B. *et al.* First human face allograft: early report. *Lancet* **368,** 203–209 (2006).

34. Lewis, J. K. *et al.* The Grand Challenges of Organ Banking: Proceedings from the first global summit on complex tissue cryopreservation. *Cryobiology* **72,** 169–182 (2016).

35. Machuca, T. N. & Cypel, M. Ex vivo lung perfusion. *J. Thorac. Dis.* **6,** 1054–62 (2014).

36. Lv, X., Tan, J., Liu, D., Wu, P. & Cui, X. Intratracheal administration of p38α short-hairpin RNA plasmid ameliorates lung ischemia-reperfusion injury in rats. *J. Heart Lung Transplant.* **31,** 655–62 (2012).

37. Whitson, B. A. & Black, S. M. Organ assessment and repair centers: The future of transplantation is near. *World J. Transplant.* **4,** 40–2 (2014).

38. Centers for Disease Control and Prevention (CDC). HIV transmitted from a living organ donor--New York City, 2009. *MMWR. Morb. Mortal. Wkly. Rep.* **60,** 297–301 (2011).

39. Fishman, J. A. & Grossi, P. A. Donor-derived infection—the challenge for transplant safety. *Nat. Rev. Nephrol.* **10,** 663–672 (2014).

40. Ward, A., Klassen, D. K., Franz, K. M., Giwa, S. & Lewis, J. K. Social, economic, and policy

implications of organ preservation advances. *Curr. Opin. Organ Transplant.* **23,** 336–346 (2018).

41. The cryonics dilemma: will deep-frozen bodies be fit for new life? | Science | The Guardian. Available at: https://www.theguardian.com/science/2016/nov/18/the-cryonics-dilemma-will-deep-frozen-bodies-be-fit-for-new-life. (Accessed: 23rd September 2018)

42. Jing, L., Yao, L., Zhao, M., Peng, L. & Liu, M. Organ preservation: from the past to the future. *Acta Pharmacol. Sin.* 1–13 (2018). doi:10.1038/aps.2017.182

43. Experiments on the principle of life, and particularly on the principle of the motions of the heart, and on the seat of this principle: including the report made to the first class of the Institute, upon the experiments relative to the mtions of the heart. (1813). Available at: https://collections.nlm.nih.gov/bookviewer?PID=nlm:nlmuid-2561017R-bk#page/1/mode/2up. (Accessed: 23rd September 2018)

44. Boettcher, W., Merkle, F. & Weitkemper, H.-H. History of extracorporeal circulation: the conceptional and developmental period. *J. Extra. Corpor. Technol.* **35,** 172–83 (2003).

45. Calne, R., Pegg, D., Pryse-Davies, J. & Brown, F. Renal preservation by ice-cooling: an experimental study relating to kidney transplantation from cadavers. *Br. Med. J.* **2,** 651–5 (1963).

46. Niemann, C. U. *et al.* Therapeutic Hypothermia in Deceased Organ Donors and Kidney-Graft Function. *N. Engl. J. Med.* **373,** 405–414 (2015).

47. Berendsen, T. A. *et al.* Supercooling enables long-term transplantation survival following 4 days of liver preservation. *Nat. Med.* **20,** 790–793 (2014).

48. Messer, S., Ardehali, A. & Tsui, S. Normothermic donor heart perfusion: current clinical experience and the future. *Transpl. Int.* **28,** 634–642 (2015).

49. Bruinsma, B. G. *et al.* Metabolic profiling during ex vivo machine perfusion of the human liver. *Sci. Rep.* **6,** 22415 (2016).

50. Storey, K. B. & Storey, J. M. Metabolic rate depression: the biochemistry of mammalian hibernation. *Adv. Clin. Chem.* **52,** 77–108 (2010).

51. Blackstone, E., Morrison, M. & Roth, M. B. H2S Induces a Suspended Animation-Like State in Mice. *Science (80-.).* **308,** 518–518 (2005).

52. Donnez, J. & Dolmans, M.-M. Ovarian cortex transplantation: 60 reported live births brings the success and worldwide expansion of the technique towards routine clinical practice. *J. Assist. Reprod. Genet.* **32,** 1167–1170 (2015).

53. Arav, A., Friedman, O., Natan, Y., Gur, E. & Shani, N. Rat Hindlimb Cryopreservation and Transplantation: A Step Toward "Organ Banking". *Am. J. Transplant.* **17,** 2820–2828 (2017).

54. Fahy, G. M. *et al.* Physical and biological aspects of renal vitrification. *Organogenesis* **5,** 167–75 (2009).

55. Song, Y. C., Khirabadi, B. S., Lightfoot, F., Brockbank, K. G. M. & Taylor, M. J. Vitreous cryopreservation maintains the function of vascular grafts. *Nat. Biotechnol.* **18,** 296–299 (2000).

56. Fuller, B. J., Lane, N. & Benson, E. E. *Life in the frozen state.* (CRC Press, 2004).

57. Jomha, N. M. *et al.* Vitrification of intact human articular cartilage. *Biomaterials* **33,** 6061–6068 (2012).

58. Pradzynski, C. C., Forck, R. M., Zeuch, T., Slavicek, P. & Buck, U. A Fully Size-Resolved Perspective on the Crystallization of Water Clusters. *Science (80-.).* **337,** 1529–1532 (2012).

59. Scambos, T. A. *et al.* Ultralow Surface Temperatures in East Antarctica From Satellite Thermal Infrared Mapping: The Coldest Places on Earth. *Geophys. Res. Lett.* **45,** 6124–6133 (2018).

60. Sformo, T. *et al*. Deep supercooling, vitrification and limited survival to -100 C in the Alaskan beetle Cucujus clavipes puniceus (Coleoptera: Cucujidae) larvae. *J. Exp. Biol.* **213,** 502–509 (2010).

61. Gertrudes, A. *et al*. How Do Animals Survive Extreme Temperature Amplitudes? The Role of Natural Deep Eutectic Solvents. *ACS Sustain. Chem. Eng.* **5,** 9542–9553 (2017).

62. Jackson, C. L. & McKenna, G. B. The melting behavior of organic materials confined in porous solids. *J. Chem. Phys.* **93,** 9002–9011 (1990).

63. Moura Ramos, J. J. & Diogo, H. P. Are Crystallization and Melting the Reverse Transformation of Each Other? *J. Chem. Educ.* **83,** 1389 (2006).

64. Mehl, P. M. Nucleation and Crystal Growth in a Vitrification Solution Tested for Organ Cryopreservation by Vitrification. *Cryobiology* **30,** 509–518 (1993).

65. Fahy, G. M., MacFarlane, D. R., Angell, C. A. & Meryman, H. T. Vitrification as an approach to cryopreservation. *Cryobiology* **21,** 407–26 (1984).

66. Callister, W. & Rethwisch, D. *Materials science and engineering: an introduction*. *Materials Science and Engineering* **94,** (2007).

67. Takahashi, T., Hirsh, A., Erbe, E. & Williams, R. J. Mechanism of cryoprotection by extracellular polymeric solutes. *Biophys. J.* **54,** 509–518 (1988).

68. Fahy, G. M. & Wowk, B. Principles of cryopreservation by vitrification. *Methods Mol. Biol.* **1257,** 21–82 (2015).

69. Hubel, A. *Preservation of Cells: a practical manual*. (Wiley/Blackwell (10.1111), 2018).

70. Baust, J. G. & Baust, J. M. Advances in biopreservation. *Adv. Biopreservation* 63 p. (2007).

71. Argüelles, J. C. Physiological roles of trehalose in bacteria and yeasts: a comparative analysis. *Arch. Microbiol.* **174,** 217–24 (2000).

72. Samali, A. & Orrenius, S. Heat shock proteins: regulators of stress response and apoptosis. *Cell Stress Chaperones* **3,** 228–36 (1998).

73. Bhattacharya, S. & Prajapati, B. G. A Review on Cryoprotectant and its Modern Implication in Cryonics. *Asian J. Pharm.* **10,** 1–7 (2016).

74. Schopp, I., Reissberg, E., Lüer, B., Efferz, P. & Minor, T. Controlled Rewarming after Hypothermia: Adding a New Principle to Renal Preservation. *Clin. Transl. Sci.* **8,** 475–478 (2015).

75. Kalogeris, T., Baines, C. P., Krenz, M. & Korthuis, R. J. Cell Biology of Ischemia/Reperfusion Injury. in *International review of cell and molecular biology* **298,** 229–317 (2012).

76. Bolli, R. & Marbán, E. Molecular and Cellular Mechanisms of Myocardial Stunning. *Physiol. Rev.* **79,** 609–634 (1999).

77. Polge, C., Smith, A. & Parkes, A. Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature* **164,** 666 (1949).

78. Jang, T. H. *et al*. Cryopreservation and its clinical applications. *Integr. Med. Res.* **6,** 12–18 (2017).

79. 21st Century Medicine --Living Tissue Preservation Solutions. Available at: http://www.21cm.com/solutions.html. (Accessed: 23rd September 2018)

80. J., C., Doshi, M. & N., R. Ice Recrystallization Inhibitors: From Biological Antifreezes to Small Molecules. in *Recent Developments in the Study of Recrystallization* (InTech, 2013). doi:10.5772/54992

81. Kim, H. J. *et al*. Marine antifreeze proteins: Structure, function, and application to cryopreservation

as a potential cryoprotectant. *Mar. Drugs* **15,** (2017).

82. Cunha, A. T. M. *et al.* Bovine epididymal spermatozoa: Resistance to cryopreservation and binding ability to oviductal cells. *Cryobiology* **73,** 348–355 (2016).

83. Fahy, G. M. The relevance of cryoprotectant toxicity to cryobiology. *Cryobiology* **23,** 1–13 (1986).

84. Fahy, G. M. Cryoprotectant toxicity neutralization. *Cryobiology* **60,** S45–S53 (2010).

85. Leibo, S. P. & Mazur, P. The role of cooling rates in low-temperature preservation. *Cryobiology* **8,** 447–452 (1971).

86. Fahy, G. M., Wowk, B., Wu, J. & Paynter, S. Improved vitrification solutions based on the predictability of vitrification solution toxicity. *Cryobiology* **48,** 22–35 (2004).

87. Notman, R., Noro, M., O'Malley, B. & Anwar, J. Molecular Basis for Dimethylsulfoxide (DMSO) Action on Lipid Membranes. *J. Am. Chem. Soc.* **128,** 13982–13983 (2006).

88. Indiveri, C., Tonazzi, A., Dierks, T., Krämer, R. & Palmieri, F. The mitochondrial carnitine carrier: characterization of SH-groups relevant for its transport function. *Biochim. Biophys. Acta - Bioenerg.* **1140,** 53–58 (1992).

89. Voigt, M. R. & DeLario, G. T. Perspectives on Abdominal Organ Preservation Solutions: A Comparative Literature Review. *Prog. Transplant.* **23,** 383–391 (2013).

90. Omasa, M. *et al.* Application of ET-Kyoto solution in clinical lung transplantation. *Ann. Thorac. Surg.* **77,** 338–339 (2004).

91. HypoThermosol® FRS | STEMCELL Technologies. Available at: https://www.stemcell.com/hypothermosolr-frs.html. (Accessed: 23rd September 2018)

92. Eagle, H. Nutrition needs of mammalian cells in tissue culture. *Science* **122,** 501–14 (1955).

93. Laing, R. W. *et al.* The Use of an Acellular Oxygen Carrier in a Human Liver Model of Normothermic Machine Perfusion. *Transplantation* **101,** 2746–2756 (2017).

94. Andrews, P. M. & Bates, S. B. Improving Euro-Collins flushing solution's ability to protect kidneys from normothermic ischemia. *Miner. Electrolyte Metab.* **11,** 309–13 (1985).

95. Karam, G. *et al.* A single solution for multiple organ procurement and preservation. *Transpl. Int.* **18,** 657–663 (2005).

96. Okada, Y. & Kondo, T. Preservation solution for lung transplantation. *Gen. Thorac. Cardiovasc. Surg.* **57,** 635–639 (2009).

97. Latchana, N., Peck, J. R., Whitson, B. & Black, S. M. Preservation solutions for cardiac and pulmonary donor grafts: a review of the current literature. *J. Thorac. Dis.* **6,** 1143–9 (2014).

98. Tokunaga, Y. *et al.* Successful 20-hour rat liver preservation with chlorpromazine in sodium lactobionate sucrose solution. *Surgery* **110,** 80–6 (1991).

99. Weimar, W. *et al.* A controlled study on the effect of mannitol on immediate renal function after cadaver donor kidney transplantation. *Transplantation* **35,** 99–101 (1983).

100. Candinas, D., Largiader, F., Binswanger, U., Sutherland, D. E. R. & Schlumpf, R. A novel dextran 40-based preservation solution. *Transpl. Int.* **9,** 32–37 (1996).

101. Davies, J. W., Ricketts, C. R. & Williams, B. N. Plasma volume expansion by rapid infusion of a low molecular weight dextran. *Br. J. Pharmacol. Chemother.* **21,** 220–5 (1963).

102. Schlumpf, R. *et al.* Dextran 40 for Clinical Organ Preservation. in **22,** 61–73 (Karger Publishers, 1996).

103. Tyers, G. F., Todd, G. J., Niebauer, I. M., Manley, N. J. & Waldhausen, J. A. The mechanism of myocardial damage following potassium citrate (Melrose) cardioplegia. *Surgery* **78,** 45–53 (1975).

104. Robinson, L. A. & Harwood, D. L. Lowering the calcium concentration in St. Thomas' Hospital cardioplegic solution improves protection during hypothermic ischemia. *J. Thorac. Cardiovasc. Surg.* **101,** 314–25 (1991).

105. Baicu, S. C. & Taylor, M. J. Acid-base buffering in organ preservation solutions as a function of temperature: new parameters for comparing buffer capacity and efficiency. *Cryobiology* **45,** 33–48 (2002).

106. Ely, S. W. & Berne, R. M. Protective effects of adenosine in myocardial ischemia. *Circulation* **85,** 893–904 (1992).

107. Boudjema, K. *et al.* Effect of oxidized and reduced glutathione in liver preservation. *Transplantation* **50,** 948–51 (1990).

108. Katkov, I. I. Introduction to the field of cryobiology and overview of selected papers. *Int. J. Refrig.* **29,** 341–345 (2006).

109. Ferguson, J., Allsopp, R. H., Taylor, R. M. & Johnston, I. D. Isolation and long term preservation of pancreatic islets from mouse, rat and guinea pig. *Diabetologia* **12,** 115–21 (1976).

110. Jang, T. H. *et al.* Cryopreservation and its clinical applications. *Integr. Med. Res.* **6,** 12–18 (2017).

111. Macklis, J. D., Ketterer, F. D. & Cravalho, E. G. Temperature dependence of the microwave properties of aqueous solutions of ethylene glycol between +15 degrees C and -70 degrees C. *Cryobiology* **16,** 272–86 (1979).

112. Fahy, G. M. & Ali, S. E. Cryopreservation of the mammalian kidney. II. Demonstration of immediate ex vivo function after introduction and removal of 7.5 M cryoprotectant. *Cryobiology* **35,** 114–31 (1997).

113. Connaughton, P. J. & Lewis, F. J. Use of glycerol and chloroform in heart preservation by freezing. *Surg. Forum* **12,** 177–9 (1961).

114. KAROW, A. M., WEBB, W. R. & STAPP, J. E. Preservation of Hearts by Freezing. *Arch. Surg.* **91,** 572 (1965).

115. Moss, G. S., Reed, P. & Riddell, A. G. Observations on the effects of glycerol on the cold storage of the canine liver. *J. Surg. Res.* **6,** 147–51 (1966).

116. Wishnies, S. M. *et al.* Biotransformation activity in vitrified human liver slices. *Cryobiology* **28,** 216–26 (1991).

117. Ekins, S. *et al.* Xenobiotic metabolism in rat, dog, and human precision-cut liver slices, freshly isolated hepatocytes, and vitrified precision-cut liver slices. *Drug Metab. Dispos.* **24,** 990–5 (1996).

118. Ishihara, K., Taniguchi, H., Hara, Y., Ejiri, K. & Baba, S. Effect of cooling rate on insulin release from frozen-thawed dispersed rat islet cells. *Diabetes Res. Clin. Pract.* **6,** 243–6 (1989).

119. Sandler, S., Kojima, Y. & Andersson, A. Cryopreservation of mouse pancreatic islets. Effects of fast cooling on islet B cell function and on the outcome of islet transplantation. *Transplantation* **42,** 588–93 (1986).

120. Hullett, D. A., Bethke, K. P., Landry, A. S., Leonard, D. K. & Sollinger, H. W. Successful long-term cryopreservation and transplantation of human fetal pancreas. *Diabetes* **38,** 448–53 (1989).

121. Kneteman, N. M., Alderson, D., Scharp, D. W. & Lacy, P. E. Long-term cryogenic storage of purified adult human islets of Langerhans. *Diabetes* **38,** 386–96 (1989).

122. Yokogawa, Y., Takaki, R., Ono, J., Okeda, T. & Yamaguchi, K. Cryopreservation of pancreatic islet

cells. *J. Lab. Clin. Med.* **103,** 768–75 (1984).

123. Sugimoto, M., Maeda, S., Manabe, N. & Miyamoto, H. Development of infantile rat ovaries autotransplanted after cryopreservation by vitrification. *Theriogenology* **53,** 1093–1103 (2000).

124. Segino, M. *et al.* In vitro culture of mouse GV oocytes and preantral follicles isolated from ovarian tissues cryopreserved by vitrification. *Hum. Cell* **16,** 109–16 (2003).

125. Migishima, F. *et al.* Successful Cryopreservation of Mouse Ovaries by Vitrification. *Biol. Reprod.* **68,** 881–887 (2003).

126. Kagabu, S. & Umezu, M. Transplantation of cryopreserved mouse, Chinese hamster, rabbit, Japanese monkey and rat ovaries into rat recipients. *Exp. Anim.* **49,** 17–21 (2000).

127. Ehrlich, L., Fahy, G. M., Wowk, B., Malen, J. A. & Rabin, Y. Thermal Analyses of a Human Kidney and a Rabbit Kidney During Cryopreservation by Vitrification. *J. Biomech. Eng.* **140,** (2017).

128. Mazur, P. Theoretical and experimental effects of cooling and warming velocity on the survival of frozen and thawed cells. *Cryobiology* **2,** 181–192 (1966).

129. Pondrom, S. White House Holds Summit on Organ Transplantation. *Am. J. Transplant.* **16,** 2241–2242 (2016).

130. Kilbride, P. *et al.* Spatial considerations during cryopreservation of a large volume sample. *Cryobiology* **73,** 47–54 (2016).

131. Nwodo, U. U., Green, E. & Okoh, A. I. Bacterial exopolysaccharides: Functionality and prospects. *Int. J. Mol. Sci.* **13,** 14002–14015 (2012).

132. Schmid, J., Sieber, V. & Rehm, B. Bacterial exopolysaccharides: Biosynthesis pathways and engineering strategies. *Front. Microbiol.* **6,** 1–24 (2015).

133. Hussain, A. *et al.* Blends and composites of exopolysaccharides; properties and applications: A review. *Int. J. Biol. Macromol.* **94,** 10–27 (2017).

134. Sutherland, I. W. Novel and established applications of microbial polysaccharides. *Trends Biotechnol.* **16,** 41–46 (1998).

135. Sergushkina, M. I., Khudyakov, A. N., Polezhaeva, T. V. & Bezmeltseva, O. M. The Ability of Pectins to Modulate the Action of Glycerol In the Freezing of Nucleated Cells.

136. Kim, S. J. & Yim, J. H. Cryoprotective properties of exopolysaccharide (P-21653) produced by the Antarctic bacterium, Pseudoalteromonas arctica KOPRI 21653. *J. Microbiol.* **45,** 510–4 (2007).

137. Casillo, A. *et al.* Structure-activity relationship of the exopolysaccharide from a psychrophilic bacterium: A strategy for cryoprotection. *Carbohydr. Polym.* **156,** 364–371 (2017).

138. Polezhaeva, T. V. *et al.* Use of pectic polysaccharides for cryopreservation of biological objects. *Arch. Biol. Sci.* **66,** 1025–1033 (2014).

139. Antunes, S., Freitas, F., Sevrin, C., Grandfils, C. & Reis, M. A. M. Production of FucoPol by Enterobacter A47 using waste tomato paste by-product as sole carbon source. *Bioresour. Technol.* **227,** 66–73 (2017).

140. Torres, C. A. V. *et al.* Rheological studies of the fucose-rich exopolysaccharide FucoPol. *Int. J. Biol. Macromol.* **79,** 611–617 (2015).

141. Freitas, F. *et al.* Fucose-containing exopolysaccharide produced by the newly isolated Enterobacter strain A47 DSM 23139. *Carbohydr. Polym.* **83,** 159–165 (2011).

142. Ferreira, A. R. V. *et al.* Biodegradable films produced from the bacterial polysaccharide FucoPol. *Int. J. Biol. Macromol.* **71,** 111–116 (2014).

143. Araújo, D. *et al*. Assessment of the adhesive properties of the bacterial polysaccharide FucoPol. *Int. J. Biol. Macromol.* **92,** 383–389 (2016).

144. Torres, C. A. V. *et al*. Rheological studies of the fucose-rich exopolysaccharide FucoPol. *Int. J. Biol. Macromol.* **79,** 611–617 (2015).

145. Reis, P. Optimization of FucoPol bioreactor production and exopolysaccharide applications. (2016).

146. Fungilab. *Rotational Viscometer Instruction Manual ALPHA SERIES Rotational Viscometer*.

147. Instruments, T. *Interpretation of the Crystallization Peak of Supercooled Liquids Using Tzero DSC*. (2007).

148. Freshney, R. I. *Culture of animal cells : a manual of basic technique*. (Wiley-Liss, 1994).

149. Liu, J. *et al*. Production, characterization and antioxidant activities in vitro of exopolysaccharides from endophytic bacterium Paenibacillus polymyxa EJS-3. *Carbohydr. Polym.* **78,** 275–281 (2009).

150. Lourenço, S. C. *et al*. Using a bacterial fucose-rich polysaccharide as encapsulation material of bioactive compounds. *Int. J. Biol. Macromol.* **104,** 1099–1106 (2017).

151. Roy Chowdhury, S. *et al*. Bacterial Fucose-Rich Polysaccharide Stabilizes MAPK-Mediated Nrf2/Keap1 Signaling by Directly Scavenging Reactive Oxygen Species during Hydrogen Peroxide-Induced Apoptosis of Human Lung Fibroblast Cells. *PLoS One* **9,** e113663 (2014).

152. Taylor, M. ., Campbell, L. ., Rutledge, R. . & Brockbank, K. G. . Comparison of Unisol with Euro-Collins solution as a vehicle solution for cryoprotectants. *Transplant. Proc.* **33,** 677–679 (2001).

153. Measurement of Cell Concentration by Optical Density | The Microbiology Network: Dedicated to the improvement of regulatory science and compliance. Available at: http://www.microbiol.org/resources/monographswhite-papers/measurement-of-cell-concentration-in-suspension-by-optical-density/. (Accessed: 23rd September 2018)

154. Provost & Wallert. *Hemocytometer Cell Counting Protocol. Biochemistry and cellular physiology* (2015).

155. González-Pinzón, R., Haggerty, R. & Myrold, D. D. Measuring aerobic respiration in stream ecosystems using the resazurin-resorufin system. *J. Geophys. Res. Biogeosciences* **117,** n/a-n/a (2012).

156. Kreft, S. & Kreft, M. Quantification of dichromatism: a characteristic of color in transparent materials. *J. Opt. Soc. Am. A* **26,** 1576 (2009).

157. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65,** 55–63 (1983).

158. Pisoschi, A. M. & Negulescu, G. P. Methods for Total Antioxidant Activity Determination: A Review. *Biochem. Anal. Biochem.* **01,** (2012).

159. Sabharwal, S. S. & Schumacker, P. T. Mitochondrial ROS in cancer: initiators, amplifiers or an Achilles' heel? *Nat. Rev. Cancer* **14,** 709–721 (2014).

160. Torres, C. A. V. *et al*. Kinetics of production and characterization of the fucose-containing exopolysaccharide from Enterobacter A47. *J. Biotechnol.* **156,** 261–267 (2011).

161. Cescut, J., Fillaudeau, L., Molina-Jouve, C. & Uribelarrea, J.-L. Carbon accumulation in Rhodotorula glutinis induced by nitrogen limitation. *Biotechnol. Biofuels* **7,** 164 (2014).

162. Alves, V. D. *et al*. Rheological and morphological characterization of the culture broth during exopolysaccharide production by Enterobacter sp. *Carbohydr. Polym.* **81,** 758–764 (2010).

163. Torres, C. A. V. *et al*. Impact of glycerol and nitrogen concentration on Enterobacter A47 growth

and exopolysaccharide production. *Int. J. Biol. Macromol.* **71,** 81–86 (2014).

164. Antunes, S., Freitas, F., Alves, V. D., Grandfils, C. & Reis, M. A. M. Conversion of cheese whey into a fucose- and glucuronic acid-rich extracellular polysaccharide by Enterobacter A47. *J. Biotechnol.* **210,** 1–7 (2015).

165. Farrugia, A. Albumin Usage in Clinical Medicine: Tradition or Therapeutic? *Transfus. Med. Rev.* **24,** 53–63 (2010).

166. Tarbell, J. M., Demaio, L. & Zaw, M. M. Effect of pressure on hydraulic conductivity of endothelial monolayers: role of endothelial cleft shear stress. *J. Appl. Physiol.* **87,** 261–268 (1999).

167. Jachimska, B. & Pajor, A. Physico-chemical characterization of bovine serum albumin in solution and as deposited on surfaces. *Bioelectrochemistry* **87,** 138–146 (2012).

168. Serafini, R. *et al.* The effect of flash-freezing temperature on stallion sperm DNA structure. *Theriogenology* **95,** 113–117 (2017).

169. Thompson, M., Bonavia, A., Thompson, M., Schryver, B. & Ehrhardt, R. Bacteria Cryopreservation Protocol. *Protoc. Exch.* (2012). doi:10.1038/protex.2012.006

170. Lourenço, A. S. C. Fucopol as encapsulating matrix of bioactive compounds. (ISA/UL, 2015).

171. Ba, Y., Mao, Y., Galdino, L. & Günsen, Z. Effects of a type I antifreeze protein (AFP) on the melting of frozen AFP and AFP+solute aqueous solutions studied by NMR microimaging experiment. *J. Biol. Phys.* **39,** 131–44 (2013).

172. Bang, J. K. *et al.* Antifreeze peptides and glycopeptides, and their derivatives: potential uses in biotechnology. *Mar. Drugs* **11,** 2013–41 (2013).

173. Wunderlich, B. Theory of Cold Crystallization of High Polymers. *J. Chem. Phys.* **29,** 1395–1404 (1958).

174. Everett, D. H. The thermodynamics of frost damage to porous solids. *Trans. Faraday Soc.* **57,** 1541 (1961).

175. Molinero, V. What determines the homogeneous freezing temperature of water? in *AIP Conference Proceedings* **1527,** 83–88 (American Institute of Physics, 2013).

176. Ba, Y., Wongskhaluang, J. & Li, J. Reversible Binding of the HPLC6 Isoform of Type I Antifreeze Proteins to Ice Surfaces and the Antifreeze Mechanism Studied by Multiple Quantum Filtering–Spin Exchange NMR Experiment. *J. Am. Chem. Soc.* **125,** 330–331 (2003).

177. STEMCELL Technologies. *CryoStor ® CS2, CS5 and CS10.*

178. Woo, K. S., Kim, H. Y., Hwang, I. G., Lee, S. H. & Jeong, H. S. Characteristics of the Thermal Degradation of Glucose and Maltose Solutions. *Prev. Nutr. food Sci.* **20,** 102–9 (2015).

179. Niedz, R. P. & Evens, T. J. A solution to the problem of ion confounding in experimental biology. *Nat. Methods* **3,** 417–417 (2006).

180. Ale, M. T., Maruyama, H., Tamauchi, H., Mikkelsen, J. D. & Meyer, A. S. Fucose-containing sulfated polysaccharides from brown seaweeds inhibit proliferation of melanoma cells and induce apoptosis by activation of caspase-3 in vitro. *Mar. Drugs* **9,** 2605–2621 (2011).

181. Péterszegi, G., Robert, A. M. & Robert, L. Protection by L-fucose and fucose-rich polysaccharides against ROS-produced cell death in presence of ascorbate. *Biomed. Pharmacother.* **57,** 130–3

182. Elmagirbi, A., Sulistyarti, H. & Atikah, A. Study of Ascorbic Acid as Iron(III) Reducing Agent for Spectrophotometric Iron Speciation. *J. Pure Appl. Chem. Res.* **1,** 11–17 (2012).

183. Prinz, H. Hill coefficients, dose-response curves and allosteric mechanisms. *J. Chem. Biol.* **3,** 37–44

(2010).

184. Adesulu-Dahunsi, A. T., Sanni, A. I. & Jeyaram, K. Production, characterization and In vitro antioxidant activities of exopolysaccharide from Weissella cibaria GA44. *LWT - Food Sci. Technol.* **87,** 432–442 (2018).

185. Liang, T.-W., Tseng, S.-C. & Wang, S.-L. Production and Characterization of Antioxidant Properties of Exopolysaccharide(s) from Peanibacillus mucilaginosus TKU032. *Mar. Drugs* **14,** (2016).

186. Liu, J. *et al*. Production, characterization and antioxidant activities in vitro of exopolysaccharides from endophytic bacterium Paenibacillus polymyxa EJS-3. *Carbohydr. Polym.* **78,** 275–281 (2009).

187. Bissoyi, A., Nayak, B., Pramanik, K. & Sarangi, S. K. Targeting Cryopreservation-Induced Cell Death: A Review. *Biopreserv. Biobank.* **12,** 23–34 (2014).

188. Miller, C. Current issues in organ preservation. in *Liver Transplantation* (2009).

189. Cheever, B. M., Master, A. & Versteegen, R. A Method for Differentiating Fetal Bovine Serum from Newborn Calf Serum. *Bioprocess. J.* 1–6 (2017).

190. Şimşek, T., Şimşek, H. U. & Cantürk, N. Z. Response to trauma and metabolic changes: posttraumatic metabolism. *Ulus. cerrahi Derg.* **30,** 153–9 (2014).

191. Szurek, E. A. & Eroglu, A. Comparison and Avoidance of Toxicity of Penetrating Cryoprotectants. *PLoS One* **6,** e27604 (2011).

192. Nalgene “Mr. Frosty” Freezing Containers. Available at: https://www.daigger.com/nalgene-mr-frosty-freezing-containers-i-nal5100-0001. (Accessed: 23rd September 2018)

193. Kilbride, P. *et al*. A scale down process for the development of large volume cryopreservation. *Cryobiology* **69,** 367–375 (2014).

194. TermoFisher Scientific. Flow Cytometry Multiplexing Used to Analyze Metabolic Activity and Assess Pharmaceutical Compound Toxicity as A High-Throughput Screening Tool - PT.

195. Martin, S. J. *et al*. Early Redistribution of Plasma Membrane Phosphatidylserine Is a General Feature of Apoptosis Regardless of the Initiating Stimulus: Inhibition by Overexpression of Bcl-2 and Abl. *J. Exp. Med.* **182,** 1545–1556 (1995).

196. Stochaj, U. *et al*. Detecting changes in the mitochondrial membrane potential by quantitative fluorescence microscopy. *Protoc. Exch.* (2015). doi:10.1038/protex.2015.009

197. Topcu, Z. Densitometric quantification of DNA topoisomers in ethidium bromide-stained agarose gels and chemiluminescence-detected X ray films. *Acta Biochim. Pol.* **47,** 835–9 (2000).

198. Bunesova, V., Lacroix, C. & Schwab, C. Fucosyllactose and L-fucose utilization of infant Bifidobacterium longum and Bifidobacterium kashiwanohense. *BMC Microbiol.* **16,** 248 (2016).

199. Lademann, J. *et al*. Penetration of titanium dioxide microparticles in a sunscreen formulation into the horny layer and the follicular orifice. *Skin Pharmacol. Appl. Skin Physiol.* **12,** 247–56 (1999).

200. Maipas, S. & Nicolopoulou-Stamati, P. Sun lotion chemicals as endocrine disruptors. *Hormones (Athens).* **14,** 32–46