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MARINE FISH ANTIFREEZE PROTEINS: THE KEY TOWARDS CRYOPRESERVING THE WINTER SOLDIER

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ABSTRACT

Along the path to building knowledge and developing new technologies, scientific research is a fountain of reliable information that allows us to probe how entities in the natural world are likely to behave. For instance, several marine fish that inhabit sub-zero temperature waters use ice-binding proteins to prevent damage to their cells and tissues. The antifreeze properties of these ice-binding proteins might hold the key for future cryopreservation applications. The non-profit organization Organ Preservation Alliance (OPA) declared that one-fifth of donated kidneys are wasted and approximately two-thirds of donated hearts and lungs never make it to a patient. Advances in cryopreservation methods would enable us to significantly reduce this donor organs wastage – possibly saving the lives of more than 110,000 people who are currently on the organ transplant waiting list in the Unites States. The establishment of a large organ library could potentially lower the patient rejection response to the transplanted organ since a more suitable donor match would be feasible. In this article, we will give an insight on the different types of ice-binding proteins, their antifreeze properties and their potential role in the cryopreservation of organs while using the fictional character known as the Winter Soldier in the Marvel Cinematic Universe as our main protagonist.

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PROLOGUE

An icy cold chill runs down his back. Flashes of scattered memories rush his brain like shards of broken glass. His hearing sharpens, strange sounds and scents excite his senses while his eyes struggle to focus and scan over his all-to-familiar confinement capsule. A freezing sensation fills up his lungs reminding him to take one last gasp of the air. That icy cold air he knows all too well.

INTRODUCTION

James Buchanan "*Bucky''* Barnes is a fictional superhero/supervillain character in the Marvel Cinematic Universe (MCU). Also known as the Winter Soldier, he is able to withstand the biological impairment of cryogenic freezing. Thus far, Barnes has appeared in three films in the MCU - *Captain America: The First Avenger* [1], *Captain America: The Winter Soldier* [2], and *Captain America: Civil War* [3]. In the latter two films, Barnes masquerades as the villainous, yet repentant, Winter Soldier. He is slated to also appear in *Avengers: Infinity War* (2018) [4] and the Avengers sequel in 2019. Although the character first appeared in the Marvel Comic books in *Captain America Comics #1* (1941) [5], he first took on the mantle of the Winter Soldier in *Captain America #1*, which was published in January 2005 [6]. In *Captain America #1*, Barnes is depicted as having been frozen for several decades after his supposed death near the end of World War II. He was only thawed, and subsequently refrozen, when his combat skills were needed for HYDRA assassination missions. After sustaining an injury during World War II, he was also fitted with a bionic arm that empowers him with superhuman strength, while his fast healing abilities and military training make him a formidable fighting weapon.

Although we are all aware that superheroes and superpowers are just fantastically unearthly figments of the creative imagination of humanity, trapped between the pages of comic books in a fictitious universe, science may be closer than you think to replicating the superpowers of characters like the Winter Soldier in the real world [7-10]. For example, there is extensive research dedicated to the development of bionic arms [11-13]. However, how likely is it that current scientific research might be able to create a real life super-soldier such as Bucky Barnes, or Captain America for that matter? Is it possible to cryogenically freeze humans, as is the case for the Winter Soldier, such that a person retains their memories and all bodily functions upon being thawed or released from cryostatus?

Cryopreservation is a process that preserves the organelles, cells, tissues or other biological material by cooling biological specimens to very low temperatures. A suitable strategy for preserving or "freezing" human tissue is still the subject of scientific investigation and review [14-17]. There are several effects of low temperature environments that can be lethal to cells and healthy tissue due to the subsequent effects of the freezing of water (Figure 1). Unprotected freezing leads to direct mechanical damage of cells due to the formation of ice crystals, which can rupture and destroy cells. In addition, secondary cellular damage is caused by extracellular ice crystal growth resulting in cellular dehydration and a rise in osmotic pressure within the cells. Excessive hyperosmotic pressure can alter membrane permeability, integrity, function and enable the diffusion of normally non-penetrating solutes across the cell membrane resulting in hyperosmotic damage of the cells [18].

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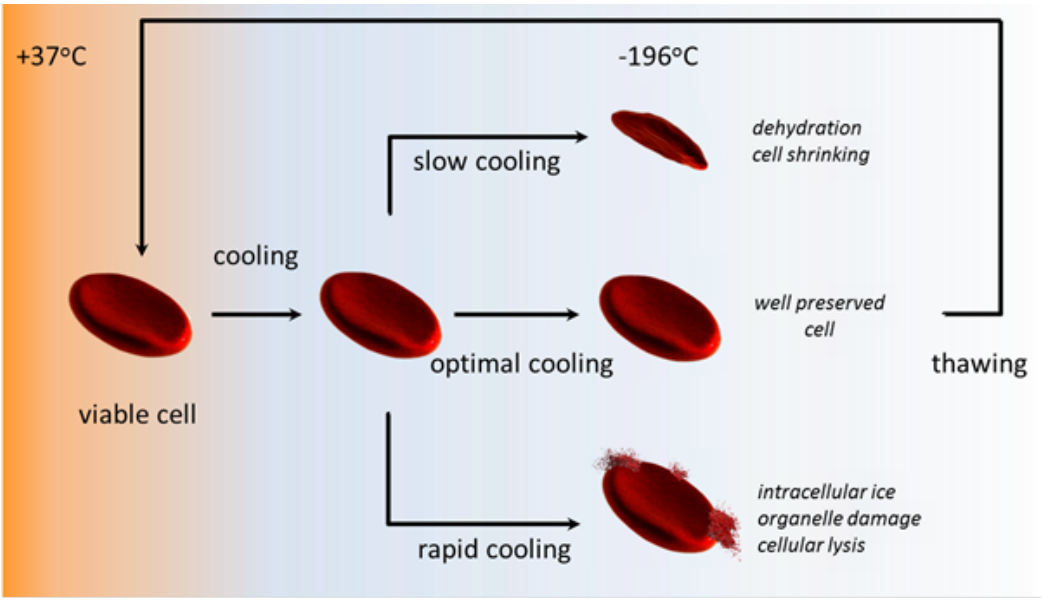

Figure 1: Schematic illustration of cryopreservation strategies differing in freezing rate and their consequences during freezing and thawing. Optimal cooling combined with the right type and amount of cryoprotectants is required to avoid cell damage due to cell shrinking and intracellular ice formation. If freezing is too slow, the ice forms extracellularly at sub-zero temperatures due to expulsion of intracellular water to the exterior. As a consequence, the cells shrink due to water loss, which triggers toxic damage related to the increase of the internal solute concentration. Fast freezing results in intracellular ice formation since it is too fast for all water molecules to escape from the cellular interior. The ice seeds that form within the cells will eventually grow (mostly during the thawing phase) due to ice recrystallization processes leading to organelle damage and cell rupture. Scheme inspired by Scott et al. (2005) [19].

The current methods for cryopreservation of donor organs mainly involve the use of cryoprotectants (CPA) and temperature control equipment. Cell penetrating CPAs such as dimethyl sulfoxide and glycerol are commonly used in cryopreservation; however, not without adverse consequences. Cell damage is not fully prevented, and the use of CPAs can be toxic to cells as a high concentration of these agents is required in the preservation process [19, 20]. Thus, the use of CPAs does not bode well for the safe preservation of large biological samples such as a person. In simple terms, freezing the Winter Soldier without taking the necessary precautions would lead to irreversible and lethal damage to his cells resulting in a heap of dead superhero tissue after resuscitation from cryostatus. Unfortunately, the Winter Soldier would be no more.

Surprisingly, the answer to the successful preservation of a real-life Bucky Barnes may have already been established in the natural world and can be specifically found in marine fish living in icy cold waters. New types of CPAs are repeatedly being investigated and a better understanding of the biology, chemistry and physics behind freezing and thawing is necessary for the development of safe cryopreservation methods [19]. Due to their non-toxicity and their remarkable antifreeze properties, ice-binding proteins (IBPs) offer a promising candidate for future cryopreservation applications, such as creating a real-life Bucky Barnes.

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WE ARE PROTEIN FACTORIES

Before we discuss the exciting subject of ice-binding proteins and their possible role in the future preservation of a real-life Winter Soldier, we will briefly describe how the human body, and the body of a fish for that matter, builds proteins. These are a subclass of polymers (from the Greek *poly* which means 'many' and *mer* which means 'parts'), which are very large molecules, also known as macromolecules, composed of many repeated subunits. Biological polymers, in short biopolymers, like deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), proteins, and polysaccharides are composed of nucleotides, amino acids or sugars. The nucleotides contain one of four nucleobases; cytosine (C), guanine (G), adenine (A) and thymine (T) [7, 21].

The genetic instructions for growth, development, functioning and reproduction of all known living organisms are stored on strings of DNA, two of which are often coiled around each other in an *antiparallel* manner to form a DNA double helix (Figure 2(a)). To produce proteins, this genetic information must be replicated and read. The enzyme RNA synthetase first copies the code onto a single strand of messenger RNA (mRNA) during 'transcription'. Each triplet of nucleotides (known as *codons*) on this mRNA encodes for one of the 20 amino acid building blocks of proteins. During 'translation', proteins are synthesized in the ribosome where the right amino acids are taxied by transfer RNA to their destination, or correct codon, and linked together into the growing protein chain. (Figure 2(c)). Curiously, this chain of amino acids then folds like biological origami into extraordinary three-dimensional shapes, such as alpha-helices and beta-sheets [21].

Every protein fulfils one or more biological function, which originates from its precise amino acid sequence and three-dimensional fold. It would be remarkable if one of our proteins would be able to repair or avoid cellular damage derived from ice crystal growth. Unfortunately, human DNA does not contain the instructions or genes for the construction of such proteins. However, certain marine fish living in icy waters do have ice-binding proteins (IBPs) with anti-freeze properties, which prevents the fish blood from freezing in the ice-cold seawaters.

WHAT ARE ICE-BINDING PROTEINS (IBPS)?

Over the course of millions of years of evolution, Nature has developed different strategies to avoid cryoinjury in organisms inhabiting cold environments. One of these strategies involves IBPs, a collection of natural cryoprotectants that allow species to survive sub-zero temperatures [22, 23]. The primary role of IBPs is to avoid ice crystal growth by binding to the surface of a small ice crystal to block further growth of the crystal. The exact details of the interaction between IBPs and ice remain an active area of investigation. The adhesion of IBPs to growing ice crystals is clearly related to the composition and structure of the region that binds to ice and the water molecules that are surrounding it. This region must match the lattice spacing of the surface ('face') of the ice crystals [23].

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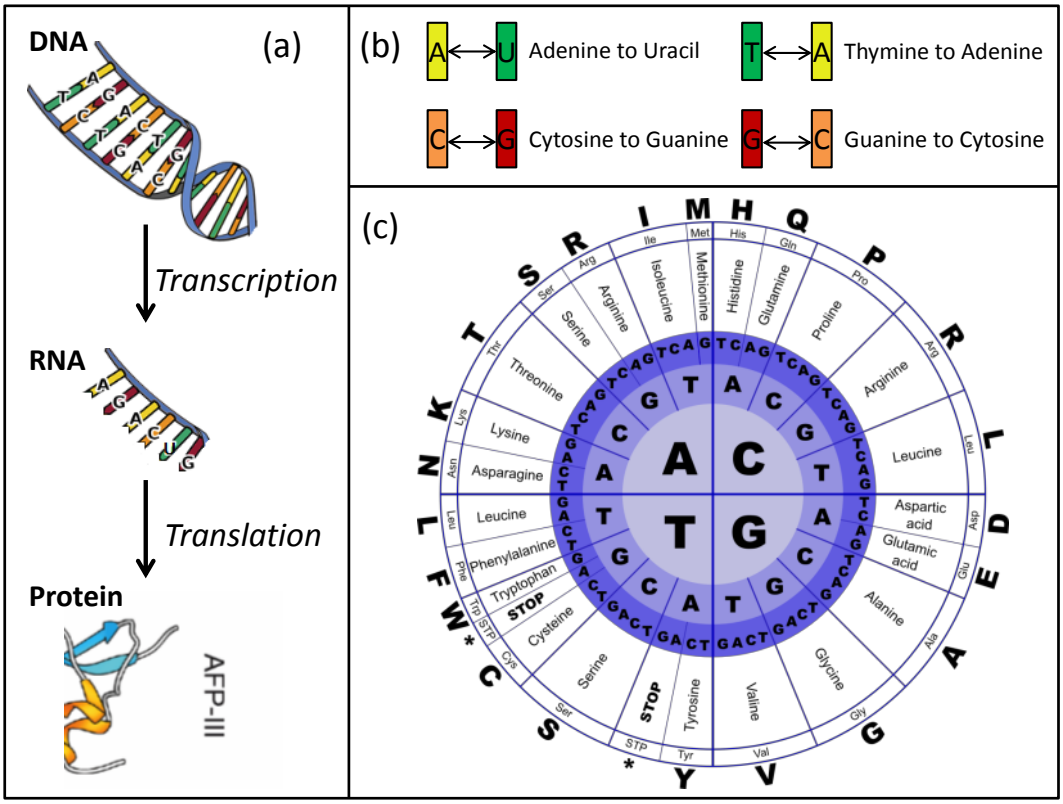

Figure 2: (a): Central dogma of molecular biology proposed by Francis Crick in 1958, which describes the steps to produce proteins from DNA. The protein RNA synthetase recognizes DNA strands carrying the genetic information for protein production and transfers the information from DNA into RNA in a process called transcription. RNA is then used by molecular machines called ribosomes to produce proteins in a process called translation. (b): Transcription base pairing rules. The DNA strand carrying biological information is transcribed using new coupling rules that pair adenine (A) with uracil (U), thymine (T) with adenine (A), cytosine (C) with guanine (G) and guanine with cytosine (C) during mRNA synthesis. (c): Codons, triplets of nucleotides matching the mRNA sequence, are read in the ribosome in the right order (from the centre to the outer part of the circle) to join the correct amino acid with the growing protein chain.

IBPs were first discovered in species of Arctic fish [24, 25], but have also been recently isolated from plants, insects, arthropods and microorganisms such as fungi, bacteria and algae [26-31]. Despite a large variation in size and structure of the proteins, all IBPs can bind to ice crystals (Figure 3). This suggests an independent and recent adaptive evolution of the protein functionality that might be due to climate change [23, 32]. IBPs from marine fish can be divided into two groups, namely: antifreeze glycoproteins (AFGPs) and antifreeze proteins (AFPs) types I to III.

FISH ANTIFREEZE GLYCOPROTEINS (AFGPS) AND ANTIFREEZE PROTEINS (AFPS)

First, we consider antifreeze glycoproteins. AFGPs are typically unordered structures that are categorized based on their size into eight classes of isoforms ($AFGP_1$ – $AFGP_8$). Of these eight isoforms, $AFGP_1$ has the highest molecular weight and consists of more than 50 repeating units, while $AFGP_8$ contains only 4 repeating units. The antifreeze activity of AFGPs

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is proportional to their molecular weight, where the largest protein has a two-fold higher activity than the smallest protein [33]. This is because a larger number of repeating units may allow for better coverage of the ice crystal surface, leading to a more efficient inhibition of ice crystal growth [26, 33]. AFGPs contain a three amino acid (alanine – alanine – threonine) repeating motif. The threonine amino acids in these proteins are attached to sugars that are critical for ice-binding, since either removing or sterically hindering the sugar moieties decreases antifreeze activity [29].

Next we focus on antifreeze proteins (AFPs). Figure 3 shows four structurally distinct fish AFPs without sugars. Flounder and sculpin fish species possess type I AFPs containing short repetitive alanine-rich sequences, which form α-helical shapes or conformations. The total amino acid sequence can contain more than 60% alanine residues, which are the elements that separate the threonine amino acids spread throughout the AFPs in repeating units of 11 amino acids. This α-helical structure has amphiphilic properties, which means that the AFPs have regions that are water-loving (hydrophilic) and water-hating (hydrophobic) [34]. Although the type I AFPs are generally small single chain proteins, there are exceptions to every rule. A larger version of the type I AFP, which is 195 amino acids in length, was discovered in the winter flounder species and has a significantly higher antifreeze activity than its smaller variant [35]. Simulations and experiments on type I AFPs in the presence of ice crystals suggest it is critical that the position of the threonine residues matches the ice lattice [36]. The alanine-rich repeats ensure a regular spacing between threonine residues, which is vital for antifreeze activity and also provide helix stabilization [37, 38].

Certain arctic species of sea ravens, smelts and herrings produce medium sized globular type II AFPs. Here, globular means that these proteins have a relatively compact folded structure that resembles a sphere. The type II AFPs need the presence of calcium to be active and contain many cysteine residues, which enhance their structural stability [39, 40]. Type II AFPs consist of two α-helices and nine β-strands that have evolved from lectin-like calcium-dependant proteins [40]. This hypothesis is supported by numerous similarities in amino acid sequence and the loss of activity of both type II AFPs and lectin-like proteins upon mutations in this region(s).

Unlike the cysteine-rich type II antifreeze proteins, type III AFPs are small globular proteins lacking cysteine residues, and can be found in the Antarctic eelpout and wolf fish. Type III AFPs can be divided into two separate groups depending on the amino acid sequences and the filtration systems used for their purification [41]. Although type III AFPs are mainly composed of several loops or coils, they form stable structures through interactions in the protein core [42, 43]. Remarkably, type III AFPs are active in a broad pH range spanning acidic to basic conditions (pH = 2-11) [43, 44]. By contrast, most other proteins partially unfold as pH values move away from the pH values of their natural environment [43]. Threonine amino acids located on the flat protein surface are thought to be crucial for the interactions between type III AFPs and ice crystals, since removal of these threonines leads to a clear loss of antifreeze activity [43, 45].

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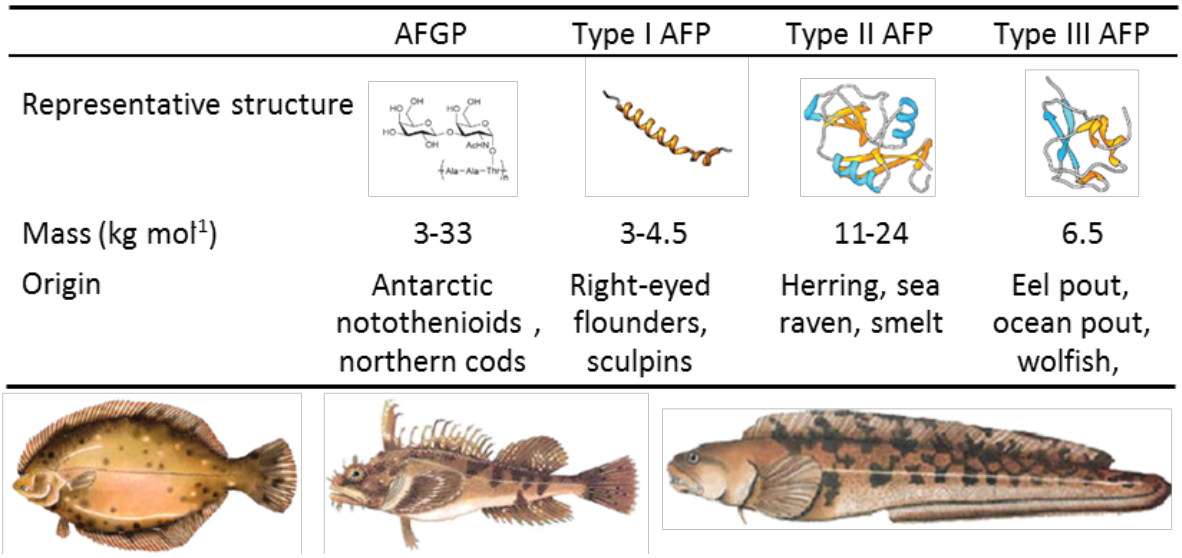

Figure 3: Overview of the size and structure of ice-binding proteins present in fish. The secondary structure of the proteins is indicated as follows: α-helix (orange), β-strand (blue), coil (grey). Representation of three different fishes with antifreeze proteins. From left to right: winter flounder (Pseudopleuronectes americanus), arctic herring (Clupea harengus), and ocean pout (Zoarces americanus). Image modified from Oude Vrielink, et al. (2016) [46].

THE BIOLOGICAL FUNCTION OF AFGPS AND AFPS

AF(G)Ps help Arctic fishes survive in their cold, ice-laden habitats in two different ways. First, both types of proteins lower the freezing temperature of water in comparison to the melting temperature, creating a temperature gap known as the thermal hysteresis (TH) gap (Figure 4(a)). In this narrow range of temperatures (down to approximately -2°C in fish serum) ice crystals do not grow nor melt [46-48]. It is well known that all solutes including salts and sugars reduce the freezing temperature of water, but only 70% of the freezing point depression of Arctic fish blood can be attributed to its salt and sugar content. The remaining 30% is attributed to the ability of AF(G)Ps to reduce the freezing point in a non-colligative manner [24]. This means that - unlike most small molecules such as alcohols, sugars and salts - the freezing point depression induced by AF(G)Ps does not increase linearly with increasing concentration. The magnitude of the TH gap induced by AF(G)Ps is used as a quantitative measure of their antifreeze activity [49, 50].

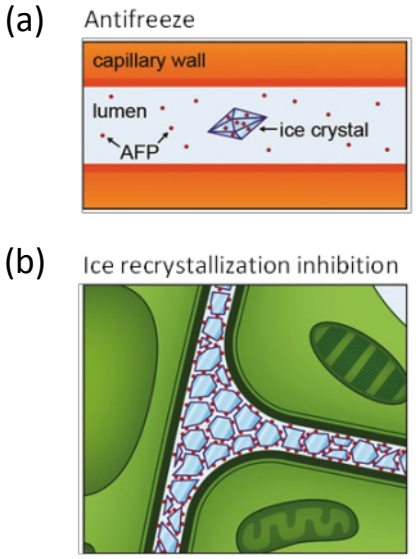

Figure 4: Fish antifreeze glycoproteins (AFGPs) and antifreeze proteins (AFPs) facilitate survival at sub-zero temperatures in ice-laden waters by (a) lowering the freezing point of Arctic fish blood and (b) inhibiting ice recrystallization. Figure from Oude Vrielink et al. (2016) [46].

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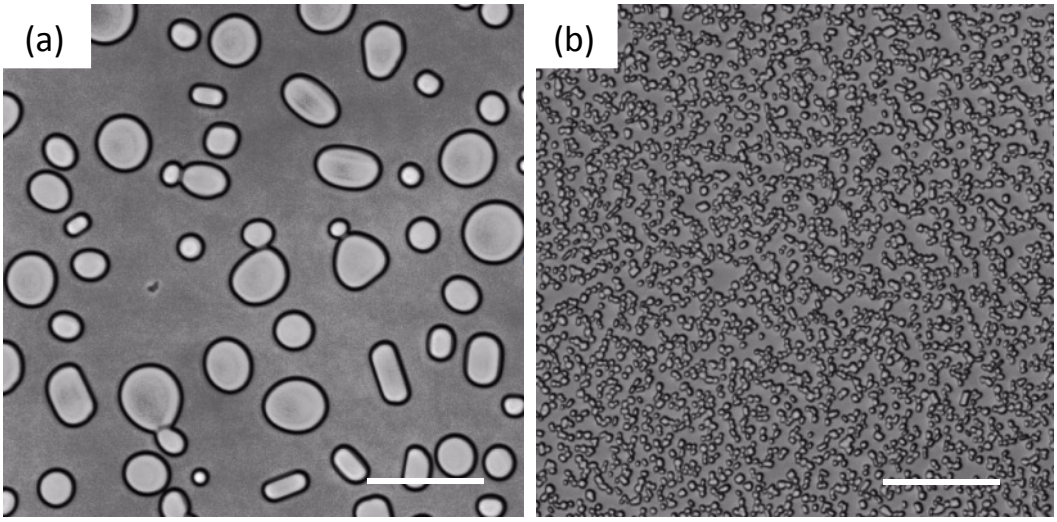

Figure 5: A sucrose sandwich assay is a test used to evaluate in a quantitative way the ice recrystallization inhibition (IRI) activity of antifreeze (glyco)proteins AF(G)Ps. Images taken one hour after the start of the IRI assay at an annealing temperature of -7 °C show (a) rather large ice crystals in a sucrose solution without AF(G)Ps, while (b) much smaller ice crystals are visible in a sucrose solution with type III AFPs at a concentration of 10 μM. The white scale bars indicate a dimension of 50μm.

The second biological function of fish AF(G)Ps, which may be more useful for cryopreservation of somebody like the Winter Soldier, is the ice recrystallization inhibition (IRI) activity (Figure 4(b)). Damage to cells is not solely caused by the formation of ice crystals, but can also be the result their growth during migratory recrystallization. This is considered the primary ice recrystallization process and it can be studied by monitoring the evolution of ice crystal size over time in a partially frozen sample (Figure 5). Large crystals grow as water molecules attach, while these detach from small crystals below a critical size, which consequently shrink and finally disappear [49, 51, 52]. In other recrystallization processes neighbouring crystals merge (accretive recrystallization) or ice crystals change their shape or internal structure (isomass recrystallization). Images taken one hour after addition of AFPs to a test solution clearly show that the antifreeze proteins hinder ice crystal growth (Figure 5). When ice formation or ingestion cannot be avoided, this ability to inhibit ice crystal growth is vital for survival. Arctic fish ingest small ice crystals throughout their lifespan as they inhabit ice-laden waters. Their AF(G)Ps block further growth of the internalized ice crystals, enabling the fish to survive despite the presence of small ice grains in their blood and in certain vital organs [22]. These intriguing cryoprotective properties make AF(G)Ps interesting candidates for utilization in cryopreservation.

COULD THE WINTER SOLDIER HAVE ANTIFREEZE PROTEINS OR ANTIFREEZE GLYCOPROTEINS IN HIS BODY?

While Arctic and Antarctic fish species naturally produce AF(G)Ps, the same cannot be said for the average person. As outlined in the section "*We are protein factories"*, we, like fish, produce

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numerous proteins every day that serve different functions. The information required for the production of these proteins is stored in our DNA as genes. For example, the gene *wf-afp* in the winter flounder, encodes for the information to produce type I AFP.

Just like Captain America, the Winter Soldier is a super-soldier who has been the subject of experimentation leading to significant changes in his DNA. It is possible that during these treatments the Winter Soldier's DNA has been adequately modified to allow his body to naturally produce the winter flounder type I AFP. As outlined in the previous section, we suggest that AF(G)Ps would be a more plausible way to improve cryopreservation given that they inhibit ice recrystallization in marine fish. It is likely that the Winter Soldier is injected with some sort of serum or medication prior to being brought in cryostatus in the 2014 film *Captain America: The Winter Soldier* [2]. This serum could contain synthetic cryoprotectants as well as an anaesthetic leading to loss of awareness and external sensation. However, in this article, we contend that following his super-soldier experimentation, the Winter Soldier's DNA has been modified to such an extent that he can naturally produce AF(G)Ps when his body is subjected to freezing temperatures.

While super-soldier serums such as those given to Captain America and the Winter Soldier are part of science fiction, a recent advancement in genetic engineering offers the possibility of accurately and safely changing human DNA in the not-too-distant-future. CRISPR/Cas9 is a genetic engineering tool that allows for precise changes to be made to the DNA of many species [53]. CRISPR/Cas9, which has been referred to as a genetic disruptor [54], effectively cuts or breaks DNA at specific locations. The DNA is cut by the Cas9 enzyme at locations that are identified using a guide RNA. The technique can be used to remove genetic code from DNA or to add new genes to DNA [55, 56].

The use of CRISPR/Cas9, or similar genetic engineering techniques, is limited to non-viable human embryo development research and is not available for the wide-scale modification of human DNA for many ethical and scientific reasons. These include, for example, potential conflicts of interests between scientific and entrepreneurial activities, unknown environmental effects and uncertain impact on future generations [57]. Nonetheless we hypothesize that the technique could be very important for the possible development of genetically advanced humans such as the Winter Soldier in future scientific laboratories. It may be possible to insert the *wf-afp* gene into human DNA using the CRISPR/Cas9, thus providing the human body with the necessary genetic code to potentially produce the wf-AFP protein. As a result, we would be able to replicate in part the Winter Soldier's ability to produce proteins to combat ice crystal growth that could arise during cryopreservation. However, giving the human body the ability to produce antifreeze proteins when in cryostatus is only part of the story. Unlike the films of the Marvel Cinematic Universe (MCU), scientists in the real world have yet to develop techniques that can resuscitate a person from cryostatus. That issue is certainly beyond the scope of this paper.

CONCLUSION

One notable feature of the eventful life of Bucky Barnes, The Winter Soldier, is that the time spent in cryostatus has had negligible effects on the functionality of his body, in particular his

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organs. Currently, research on the physical and chemical properties of cells after freeze-thawing cycles are necessary for successful cryopreservation in clinical applications. These studies are the first step towards achieving tissue and organ cryopreservation, which would be a very significant medical advance. Successful progress in this field could potentially aid the reduction of organ waste due to hypothermal damage during organ preservation.

The secret towards addressing these challenges in cell, tissue and organ cryopreservation could lie inside the DNA of Arctic marine fish. Their ability to naturally produce antifreeze proteins could hold the key towards revolutionising our methods for prolonging the lifetime of organs from hours to, potentially, forever. The prospect of successfully preserving and resuscitating humans, like the Winter Soldier, lies in the distant future. If advanced human cryopreservation methods were to come to fruition in the future, it seems highly unlikely that these methods will be used for the preservation of brainwashed deadly assassins such as the Winter Soldier. Just like Bucky Barnes may hold his breath just before being frozen, we hold our breaths in anticipation of scientific advances that could save or prolong the lives of millions. The Winter Soldier and his fractured life story could yet inspire a cryorevolution.

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REFERENCES

1. Johnston, J., *Captain America: The First Avenger (motion picture)*. 2011, Marvel Studios.
2. Russo, J. and A. Russo, *Captain America: The Winter Soldier (motion picture)*. 2014, Marvel Studios.
3. Russo, J. and A. Russo, *Captain America: Civil War (motion picture)*. 2016, Marvel Studios.
4. Russo, J. and A. Russo, *Avengers: Infinity War*. 2018, Marvel Studios.
5. Kirby, J. and J. Simon, *Captain America Comics*. 1941, Timely Publications.
6. Brubaker, E., *Captain America Vol. 1: Winter Soldier, Book One*. 2006: Marvel Comics. 168.
7. Fitzgerald, B.W., *Secrets of Superhero Science*. 2016, the Netherlands: BW Science.
8. Fitzgerald, B.W., *Using Hawkeye from the Avengers to communicate on the eye.* Advances in Physiology Education, 2018. **42**(1): p. 90-98.
9. Fitzgerald, B.W., *Using superheroes such as Hawkeye, Wonder Woman and the Invisible Woman in the physics classroom.* Physics Education, 2018. **53**(3): p. 035032.
10. Fitzgerald, B.W., *Superhero Science and Technology: A New Open Access Journal.* Superhero Science and Technology, 2018. **1**(1).
11. Bandara, D.S.V., et al., *Development of a multi-DoF transhumeral robotic arm prosthesis.* Medical Engineering and Physics. **48**: p. 131-141.
12. Hutchinson, D.T., *The Quest for the Bionic Arm.* JAAOS - Journal of the American Academy of Orthopaedic Surgeons, 2014. **22**(6): p. 346-351.
13. Seo, M., H. Kim, and Y. Choi. *Human mimetic forearm mechanism towards bionic arm*. in *2017 International Conference on Rehabilitation Robotics (ICORR)*. 2017.
14. Mocé, E., A.J. Fajardo, and J.K. Graham, *Human Sperm Cryopreservation.* EMJ, 2015. **1**(1): p. 86-91.
15. Mohr, J., et al., *Disinfection of human musculoskeletal allografts in tissue banking: a systematic review.* Cell and Tissue Banking, 2016. **17**(4): p. 573-584.

16. Singh, R., D. Singh, and A. Singh, *Radiation sterilization of tissue allografts: A review.* World Journal of Radiology, 2016. **8**(4): p. 355-369.
17. Harper, J.C., et al., - *Recent developments in genetics and medically assisted reproduction: from research to clinical applications.* 2018. **- 26**(- 1): p. - 33.
18. Mazur, P., *Freezing of living cells: mechanisms and implications.* American Journal of Physiology-Cell Physiology, 1984. **247**(3): p. C125-C142.
19. Scott, K.L., J. Lecak, and J.P. Acker, *Biopreservation of Red Blood Cells: Past, Present, and Future.* Transfusion Medicine Reviews, 2005. **19**(2): p. 127-142.
20. Bakhach, J., *The cryopreservation of composite tissues: Principles and recent advancement on cryopreservation of different type of tissues.* Organogenesis, 2009. **5**(3): p. 119-126.
21. Martin, E. and W. Saenger, *Principles of Nucleic Acid Strcuture*. 2013: Springer Berlin Heidelberg.
22. Cziko, P.A., et al., *Antifreeze protein-induced superheating of ice inside Antarctic notothenioid fishes inhibits melting during summer warming.* Proceedings of the National Academy of Sciences, 2014. **111**(40): p. 14583.
23. Davies, P.L., *Ice-binding proteins: a remarkable diversity of structures for stopping and starting ice growth.* Trends in Biochemical Sciences, 2014. **39**(11): p. 548-555.
24. Devries, A.L., *Glycoproteins as Biological Antifreeze Agents in Antarctic Fishes.* Science, 1971. **172**(3988): p. 1152.
25. DeVries, A.L. and D.E. Wohlschlag, *Freezing Resistance in Some Antarctic Fishes.* Science, 1969. **163**(3871): p. 1073.
26. Burcham, T.S., et al., *Analysis of antifreeze glycoproteins in fish serum.* Analytical Biochemistry, 1984. **139**(1): p. 197-204.
27. Gilbert, J.A., P.L. Davies, and J. Laybourn-Parry, *A hyperactive, Ca2+-dependent antifreeze protein in an Antarctic bacterium.* FEMS Microbiology Letters, 2005. **245**(1): p. 67-72.
28. Gupta, R. and R. Deswal, *Antifreeze proteins enable plants to survive in freezing conditions.* Journal of Biosciences, 2014. **39**(5): p. 931-944.
29. Harding, M.M., P.I. Anderberg, and A.D.J. Haymet, *'Antifreeze' glycoproteins from polar fish.* European Journal of Biochemistry, 2003. **270**(7): p. 1381-1392.
30. Jung, W., et al., *Isolation and Characterization of Antifreeze Proteins from the Antarctic Marine Microalga Pyramimonas gelidicola.* Marine Biotechnology, 2014. **16**(5): p. 502-512.
31. Kiko, R., *Acquisition of freeze protection in a sea-ice crustacean through horizontal gene transfer?* Polar Biology, 2010. **33**(4): p. 543-556.
32. Basu, K., et al., *Flies expand the repertoire of protein structures that bind ice.* Proceedings of the National Academy of Sciences, 2015. **112**(3): p. 737.
33. Knight, C.A., A.L. De Vries, and L.D. Oolman, *Fish antifreeze protein and the freezing and recrystallization of ice.* Nature, 1984. **308**: p. 295.
34. Sicheri, F. and D.S.C. Yang, *Ice-binding structure and mechanism of an antifreeze protein from winter flounder.* Nature, 1995. **375**: p. 427.
35. Sun, T., et al., *An Antifreeze Protein Folds with an Interior Network of More Than 400 Semi-Clathrate Waters.* Science, 2014. **343**(6172): p. 795.
36. Nada, H. and Y. Furukawa, *Antifreeze proteins: computer simulation studies on the mechanism of ice growth inhibition.* Polymer Journal, 2012. **44**: p. 690.
37. Cheng, A. and K.M. Merz, Jr., *Ice-binding mechanism of winter flounder antifreeze proteins.* Biophysical Journal. **73**(6): p. 2851-2873.
38. Baardsnes, J., et al., *New ice - binding face for type I antifreeze protein.* FEBS Letters, 1999. **463**(1-2): p. 87-91.
39. Slaughter, D., et al., *Antifreeze proteins from the sea raven, Hemitripterus americanus. Further evidence for diversity among fish polypeptide antifreezes.* Journal of Biological Chemistry, 1981. **256**(4): p. 2022-2026.
40. Ewart, K.V., et al., *The Ice-Binding Site of Atlantic Herring Antifreeze Protein Corresponds to the Carbohydrate-Binding Site of C-Type Lectins.* Biochemistry, 1998. **37**(12): p. 4080-4085.
41. Hew, C.L., et al., *Multiple genes provide the basis for antifreeze protein diversity and dosage in the ocean pout, Macrozoarces americanus.* Journal of Biological Chemistry, 1988. **263**(24): p. 12049-12055.
42. Yang, C. and K.A. Sharp, *The mechanism of the type III antifreeze protein action: a computational study.* Biophysical Chemistry, 2004. **109**(1): p. 137-148.
43. Chao, H., et al., *Structure-function relationship in the globular type III antifreeze protein: identification of a cluster of surface residues required for binding to ice.* Protein Science : A Publication of the Protein Society, 1994. **3**(10): p. 1760-1769.
44. García-Arribas, O., et al., *Thermodynamic stability of a cold-adapted protein, type III antifreeze protein, and energetic contribution of salt bridges.* Protein Science : A Publication of the Protein Society, 2007. **16**(2): p. 227-238.
45. Olijve, L.L.C., A.S. Oude Vrielink, and I.K. Voets, *A Simple and Quantitative Method to Evaluate Ice Recrystallization Kinetics Using the Circle Hough Transform Algorithm.* Crystal Growth & Design, 2016. **16**(8): p. 4190-4195.
46. Oude Vrielink, A.S., et al., *Interaction of ice binding proteins with ice, water and ions.* Biointerphases, 2016. **11**(1): p. 018906.
47. Duman, J.G., *Animal ice-binding (antifreeze) proteins and glycolipids: an overview with emphasis on physiological function.* The Journal of Experimental Biology, 2015. **218**(12): p. 1846.
48. Voets, I.K., *From ice-binding proteins to bio-inspired antifreeze materials.* Soft Matter, 2017. **13**(28): p. 4808-4823.
49. Olijve, L.L.C., et al., *Blocking rapid ice crystal growth through nonbasal plane adsorption of antifreeze proteins.* Proceedings of the National Academy of Sciences, 2016. **113**(14): p. 3740.
50. Yu, S.O., et al., *Ice restructuring inhibition activities in antifreeze proteins with distinct differences in thermal hysteresis.* Cryobiology, 2010. **61**(3): p. 327-334.
51. Budke, C., et al., *Ice Recrystallization Kinetics in the Presence of Synthetic Antifreeze Glycoprotein Analogues Using the Framework of LSW Theory.* The Journal of Physical Chemistry B, 2009. **113**(9): p. 2865-2873.
52. Budke, C., et al., *Quantitative Efficacy Classification of Ice Recrystallization Inhibition Agents.* Crystal Growth & Design, 2014. **14**(9): p. 4285-4294.
53. Doudna, J.A. and E. Charpentier, *The new frontier of genome engineering with CRISPR-Cas9.* Science, 2014. **346**(6213).

 DOI: 10.24413/SST.2018.1.2105

54. Ledford, H., *CRISPR, the disruptor.* Nature, 2015. **522**: p. 20-24.
55. Liang, P., et al., *CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes.* Protein & Cell, 2015. **6**(5): p. 363-372.
56. Tang, L., et al., *CRISPR/Cas9-mediated gene editing in human zygotes using Cas9 protein.* Molecular Genetics and Genomics, 2017. **292**(3): p. 525-533.
57. de Lecuona, I., et al., *Gene Editing in Humans: Towards a Global and Inclusive Debate for Responsible Research.* The Yale Journal of Biology and Medicine, 2017. **90**(4): p. 673-681.