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Review

# Cryopreservation of articular cartilage ☆

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ABSTRACT

Cryopreservation has numerous practical applications in medicine, biotechnology, agriculture, forestry, aquaculture and biodiversity conservation, with huge potentials for biological cell and tissue banking. A specific tissue of interest for cryopreservation is the articular cartilage of the human knee joint for two major reasons: (1) clinically, there exists an untapped potential for cryopreserved cartilage to be used in surgical repair/reconstruction/replacement of injured joints because of the limited availability of fresh donor tissue and, (2) scientifically, successful cryopreservation of cartilage, an avascular tissue with only one cell type, is considered a stepping stone for transition from biobanking cell suspensions and small tissue slices to larger and more complicated tissues. For more than 50 years, a great deal of effort has been directed toward understanding and overcoming the challenges of cartilage preservation. In this article, we focus mainly on studies that led to the finding that vitrification is an appropriate approach toward successful preservation of cartilage. This is followed by a review of the studies on the main challenges of vitrification, i.e. toxicity and diffusion, and the novel approaches to overcome these challenges such as liquidus tracking, diffusion modeling, and cryoprotective agent cocktails, which have resulted in the recent advancements in the field.



## Introduction

Articular cartilage is the white dense material covering the ends of the bones in the articulating joints, such as the knee. Compared to most other tissue types in the human body, articular cartilage is a simple tissue containing only one cell type, called chondrocytes, with no vascular, lymphatic or nervous system. Articular cartilage consists of a collagen network, predominantly of collagen type II, developed specifically to respond to the mechanical forces on the joint. Packed within this collagen network are proteoglycans that provide the hydraulic-like resistance to mechanical forces. These proteoglycans are hydrophilic resulting in a large proportion of the weight and volume of articular cartilage being water (varying from 65% to 80% depending on the type and depth of the cartilage). Chondrocytes are the lone cell type present in cartilage, and are scattered throughout the matrix with a denser, horizontally aligned distribution close to the contact surface (tangential zone). Further from the surface, the density of chondrocytes decreases and they become randomly distributed. Finally, closer to the tide-mark (bone-cartilage boundary) the cells are more vertically aligned. Chondrocytes produce and maintain the extracellular matrix precursor material including the collagen and the proteoglycans that also change in distribution and orientation from superficial to deep within the cartilage matrix. The absence of a vascular supply in articular cartilage means that the cells receive nutrients and discharge waste material by diffusion through the extracellular matrix, from and to the synovial fluid, respectively. As a result, articular cartilage has a very limited ability to self-heal and joint injuries with articular cartilage damage can lead to cartilage degeneration and subsequent osteoarthritis with significant personal and socioeconomic costs [93].

Cartilage replacement or repair can be indicated for a number of medical conditions including: avascular necrosis/osteochondritis dissecans, traumatic injuries, epiphyseal tumors and arthritis. These conditions do not have good non-surgical or surgical options to restore joint function and, if left untreated, they lead to joint instability, deterioration and subsequent osteoarthritis. Arthritis is the most common cause of disability in North America and osteoarthritis is the most common form of arthritis. More than 20 million people in the US alone deal with severe limitations in function on a daily basis due to arthritis, which results in more than 1 million hospitalization cases, and costs a total of $100 billion US every year [1].

Surgical treatment options depend on the type and size of the cartilage lesion. Small lesions less than 1 cm in diameter typically can be compensated by the surrounding cartilage but not always.

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Persistently symptomatic smaller lesions and larger lesions often require surgical intervention. The most common cell-based interventions are microfracture [97,98], autologous chondrocyte implantation (ACI) [15,16], and matrix associated ACI (MACI) [11,66]. There are many reports of these surgical techniques providing some positive results but they are unable to reproduce the complex structure of the articular cartilage matrix resulting in biomechanically inferior fibrocartilage or "hyaline-like" cartilage. It is possible that these inferior cartilage repair tissues will not function well in the long-term. Another surgical treatment option is mosaicplasty (osteochondral autografts) which consists of taking cartilage from one area of the joint and moving it to the defect. This will restore the normal cartilage matrix structure in the injured area at the cost of removing it from a previously healthy area of the joint. All of these techniques can provide some relief in small to medium size defects but they are not able to treat large defects, including whole joints, effectively. The only biologic technique that can restore partial [102,103], or whole joints [9,19,36] is osteochondral allografting [38] which entails transplanting bone and cartilage from a donor patient into a recipient patient. In older patients, synthetic total joint replacement is a viable option for more sedentary, low activity patients. Unfortunately, this is not as good an option in younger or active patients because the synthetic joint replacements tend to fail after 7–15 years. Osteochondral transplantation is a possible option for these younger, more active patients but access to fresh tissue is extremely difficult.

Fresh osteochondral transplants provide the best case scenario for cell viability and matrix integrity but fresh transplant is fraught with technical difficulties. This tissue should be harvested within 24 h of death of the donor and is typically transplanted within 48–72 h after harvest [37]. This time frame is too short to perform the extensive testing required to rule out the possibility of transmission of infectious diseases. Considering that joint injury and osteoarthritis are not life threatening, the risk may not be warranted. Another significant technical difficulty is that matching for size and contour, which are important factors for long term successful outcomes, is extremely difficult on such short notice [19]. Making arrangements for complicated joint replacement surgery on short notice can result in logistic problems in arranging the operating room, appropriate surgical staff, surgeon and even the patient. Currently, blood/HLA typing is not performed as articular cartilage is considered immune privileged. That said, the cartilage is transplanted on bone and there can be minor immune reaction to the transplanted bone. This is typically self-limited as the transplant bone is replaced with host bone if only a small amount is transplanted. In the future, blood/HLA typing may be employed to limit the immune reaction which adds another layer of complexity to performing this surgery on short notice. To address these issues, hypothermic storage at 4 °C for a limited time (28–42 days) is used to increase the supply [41,110]. Unfortunately, tissue deterioration begins after only 7–14 days [65]. The lack of normal mechanical stimulation impairs the efficiency of nutrient and waste transport, and decreases cytokine secretion (IL-1 and TNF-α) as reviewed by Kim, Teng and Dang [58]. The ability to store articular cartilage indefinitely would allow for precise size/contour matching, pre-surgical planning, testing for infectious diseases, possible blood typing and appropriate surgical timing for the patient, operating staff and surgeon.

Successful cryopreservation of articular cartilage, by either classical methods or vitrification, can extend the availability of the tissue and allow long-term banking of articular cartilage. Successful cryopreservation and banking of articular cartilage will enable easier and more efficient utilization of straightforward protocols for transplantation. From a cryopreservation perspective, articular cartilage with its extracellular matrix containing no lymphatic, nervous or vascular systems and only one cell type is considered to be a stepping stone for the transition from simple cell to complex tissue cryopreservation with high cell viability and function. Information gained from cryopreservation of articular cartilage can provide valuable insight into more complex tissue and organ cryopreservation.

In the following, a review of articular cartilage cryopreservation methods for transplantation is presented. First, the milestones of cartilage cryopreservation research are reviewed in chronological order, and the basics of associated injuries in classical cryopreservation methods for cartilage are discussed. Then, the prospect of vitrification in lieu of classical cryopreservation, and the current status of cartilage cryopreservation are reviewed. At the end, a summary of challenges are presented and viable approaches are discussed.

## Cryopreservation-associated injuries in cartilage

Successful cryopreservation of articular cartilage is difficult to achieve due to general cryopreservation challenges and some cartilage-specific challenges. Tissues are more challenging to cryopreserve than cellular systems in suspension for many reasons. In tissues, both the cellular activity and the matrix structure must be preserved and this is complicated by the intimate relationship of the cells with the extracellular matrix. Tissues generally contain multiple cell types each with different cryopreservation parameters. Furthermore, different tissues have different requirements for transplantation. In some tissues, such as skin or bone grafts, transplantation of the extracellular matrix is preferred without the native cells to decrease the risk of immunorejection in the recipient [12,42]. Alternatively, some tissues such as articular cartilage require the cellular system for proper long-term functioning of the extracellular matrix; therefore, the cryopreservation strategy must be able to minimize the damage to both the extracellular matrix and the cells.

### *Cryopreservation of isolated chondrocytes*

The earliest investigation into the preservation of chondrocytes was done by Curran and Gibson (1956) [22] who investigated the radioactive sulfate uptake of chondroitin sulfate in human chondrocytes as a measure of chondrocyte viability in 0.5 mm thick cartilage slices obtained from rib, ear or nose. They demonstrated that the cartilage can stay viable for up to 40 days in Tyrode solution at 4 °C. However, cartilage slices, untreated or pretreated (with 10% to 30% w/w glycerol solutions), cooled down to −25 °C showed no recovery of the chondrocytes. Heyner (1960) [40] trypsinized the cartilage for 25 min before slow and rapid freezing in 15% glycerol solutions. It appeared that the chondrocytes in trypsinized cartilage could survive slow freezing to −79 °C and grow in culture while the chondrocytes in untrypsinized cartilage could not tolerate freezing temperatures lower than −20 °C. It was concluded that the failure of the chondrocytes to survive freeze–thaw protocols was related to the cartilage matrix and cell-matrix interactions. Subsequent research was performed on isolated chondrocytes to determine their ability to survive freeze–thaw protocols before spending more effort on the chondrocytes *in situ*.

Unsuccessful freezing of cartilage slices by Curran and Gibson [22] (1956), followed by encouraging results from freezing of partially trypsinized cartilage by Heyner [40] (1960), led to cryopreservation of isolated chondrocytes from rabbits, dogs, monkeys and human, by Smith (1965) [94], to avoid complications of dealing with a collagen matrix. Smith replaced glycerol with $Me_2SO$ and cooled the chondrocytes in 10% w/w $Me_2SO$ to −20 °C at −1 °C/min followed by cooling at −4 °C/min to −79 °C, and found that

a large proportion of the chondrocytes from all four species maintained viability, assessed by physical appearance compared to a control group, after thaw in a +40 °C water bath. Chesterman and Smith (1968) [21] completed Smith's study by transplanting the frozen–thawed chondrocytes into cancellous bone to evaluate cell function through growth rate. The chondrocytes were able to produce a new cartilage matrix at the sites of resorption after 2 weeks. This work answered the question of whether the chondrocytes can function properly after cryopreservation. Despite this success, there were more unknowns that needed to be addressed prior to attempting to cryopreserve intact cartilage such as tolerable toxicity limits of chondrocytes.

Tomford et al. (1984) [101] isolated the chondrocytes from bovine articular cartilage to evaluate the toxicity limits of cryoprotective agents (CPA) as a function of time, temperature and the concentration of the CPA. The toxicity of $Me_2SO$ can be due to interactions with the lipid bilayer membrane of the cells [107] and the intracellular enzymes [87]. Tomford et al. (1984) also investigated the optimum cooling rates for cryopreservation of isolated bovine chondrocytes in a two stage slow- and rapid-cooling protocol following suggestions by Smith et al. (1965). In 1988, McGann et al. [67] addressed the role of cell membrane permeability to water as a key in the success of freezing protocols and combined computer simulations with physical understanding of the cell freezing process in designing cryopreservation protocols for isolated chondrocytes. His works along with others resulted in successful cryopreservation of chondrocytes in slices. A protocol of 10% w/w $Me_2SO$ with −1 °C/min slow-cooling was established for high recovery cryopreservation of isolated chondrocytes similar to many other cell types [68]. Schachar and McGann (1986) [90] reported 80–90% cell viability, assessed by membrane integrity test, for isolated chondrocytes and approximately 50% for the chondrocytes in thin slices of cartilage using 10% $Me_2SO$ and slow-cooling. With these successes, the logical next step was to apply the same protocol to full-thickness cartilage for transplantation.

*Cryopreservation of osteochondral plugs*

The discrepancy in the success rates for isolated chondrocyte and *in situ* chondrocyte cryopreservation led to studies on the effect of ice formation on the chondrocytes in the cartilage matrix. In search of an explanation for this inconsistency, Kawabe and Yoshinao (1990) [54] investigated the cryopreservation of *in vitro* rabbit articular cartilage slices using functional assays (by incorporation of $Na_2SO_4$). They determined that diffusion was a significant factor with almost double the cell viability in the group with holes created in the matrix using a needle compared to the group with normal matrix. Similar increases in cell viability were also noted after bone carrier alteration was performed by Jomha et al. [47]. Schachar et al. (1992) expanded on their success of cartilage slice cryopreservation [90] by using a rabbit model to investigate the fate of the cryopreserved chondrocytes in osteochondral allografts and autografts after transplantation [89]. They showed that the chondrocytes in osteochondral dowels cryopreserved in 7.5% $Me_2SO$ and cooled at −1 °C/min down to −70 °C maintained some functional activity although it was significantly less than the untreated control group at 12 months after transplantation. Furthermore, these cryopreserved dowels demonstrated a thinner cartilage after 12 months indicating some breakdown of the cartilage matrix suggesting that the functional activity of the chondrocytes was insufficient to maintain the matrix over the long-term. A study by Tavakol et al. (1993) [100] investigated the changes in the ultrastructure of chondrocytes *in situ* after a freeze–thaw cycle with and without $Me_2SO$ present using electron microscopy. The freeze–thaw conditions were similar to the study by Schachar et al. (1992). As expected, the chondrocytes in control samples without $Me_2SO$ were completely destroyed. Surprisingly, the chondrocytes in the samples treated with $Me_2SO$ did not maintain a significantly higher percentage of cellular structure integrity or cell-matrix junctions. Such results supported the assumption that the difficulty in cryopreservation of chondrocytes *in situ* after slow-freezing could be related to the loss of chondrocyte-matrix interaction, the altered extracellular matrix structure, and possibly the location of the chondrocytes within the matrix. Following that, Muldrew et al. (1994) [73] investigated the localization of chondrocyte viability *in situ* in osteochondral dowels in a sheep model using a graded freezing approach and 10% $Me_2SO$. Prior to this work, chondrocyte viability was determined by isolating the chondrocytes from the matrix and characterizing cell membrane integrity or biological function. This method was unable to reveal any information regarding the distribution of the damaged and intact chondrocytes within the cartilage matrix. The study by Muldrew et al. suggested that the viability of the chondrocytes was depth-dependent with the greatest damage in the middle zone. Ohlendorf et al. (1996) [79] later obtained a pattern of viable and injured cells similar to that of Muldrew et al. (1994) [73]. Both studies commented on the multiple reasons for observing such survival patterns but the main proposed reason was the association of $Me_2SO$ diffusion with the thickness of the cartilage matrix. It was suggested that biological and biophysical reasons might exist for the apparent higher susceptibility of the chondrocytes in the middle zone and that this could be investigated in more depth by modeling or measurement of the stresses in the middle zone. In 2000, Muldrew et al. published another study [75] on the ice morphology and its effect on the recovery of the chondrocytes. The results of that study suggested two mechanisms of damage to the chondrocytes and the matrix. First, the planar ice growth in the tissue is limited by the diffusion of the solutes away from the ice front. This can cause supercooling in the tissue and perhaps spontaneous ice nucleation within the lacunae. Second, ice formation can mechanically crush the cells, expand the pore size and disrupt the matrix, as was demonstrated by scanning electron microscopy from frozen specimens. It was hypothesized that the damage to the chondrocytes could be in part due to ice formation in the lacunae where large amounts of water exists compared to within porosities of the collagen matrix (capillaries). Liu et al. showed that solutions in cartilage capillaries have lower freezing points than in larger spaces, and ice formation always starts from larger spaces within cartilage [63]. In 2001, Muldrew et al. showed that it is possible to achieve high recovery of the chondrocytes in ovine cartilage grafts using a 2-step cooling method [74]. However, large acellular regions and multicellular clumps of chondrocytes were observed in the transplanted articular cartilage 3–12 months after transplantation, suggesting an unknown type of cryoinjury [72]. Unfortunately, the high cell recovery of this 2-step cooling method was not reproducible when using thicker human articular cartilage [50].

The effect of ice formation and vitrification on the cartilage matrix has been investigated. Laouar et al. demonstrated microstructural changes due to ice formation in the cartilage matrix after $Me_2SO$ slow-cooling cryopreservation using MRI and biochemical analysis. Some protection was noted by the use of $Me_2SO$ [60]. Jomha et al. showed significantly more ice formation using lower concentrations of $Me_2SO$ (1 M) when compared to vitrifiable concentrations (6 M) where minimal matrix distortion was noted [48]. Further evidence for matrix damage was provided by Pegg et al. [82–84] in a series of comprehensive studies using the liquidus-tracking method previously introduced by Farrant in 1965 [33] for cellular cryopreservation in suspension. The initial study demonstrated ~56% recovery of chondrocyte viability and function in 0.7 mm thick discs of ovine articular cartilage cut from the bone

[82]. Later, the technique was improved by automation of liquidus-tracking vitrification of cartilage dowels increasing the recovery to 87% in the same ovine discs as the initial study [106]. According to this study, ice formation within the chondrons was the major cause of cell viability loss during cryopreservation. For a detailed analysis of the damage mechanisms in frozen articular cartilage, see the study by Pegg et al. [83].

From a clinical perspective, Song et al. (2004) evaluated the response of vitrified cartilage grafts vs. slow-cooled grafts in rabbits and concluded that the vitrified grafts performed significantly better than the nonvitrified group [95]. The results of these studies along with previous observations by Tavakol et al. (1993) and Muldrew et al. (2000) suggested that vitrification may be advantageous for preservation of cartilage over traditional slow-freezing. It is evident now that ice formation damages the matrix and alters cartilage mechanical properties through breakage and fragmentation of ECM components including fibronectin which can start the cascade of cellular injury by interacting with cell surface integrins and stimulate production of matrix-degrading proteinases [35].

An alternative method of articular cartilage cryopreservation is classical slow-cooling cryopreservation of cartilage using directional freezing [8]. This technique is based on the assumption that uncontrolled ice crystal formation and propagation within the tissue is the major cause of damage, presumably due to mechanical crushing and electrolyte concentration. Norman et al. controlled the rate of freezing and planar ice front propagation in porcine cartilage plugs using a state-of-the-art temperature-control system [77]. They reported cartilage health in terms of cell viability (53% membrane integrity), functional assays (59% $^{35}SO_4$ uptake) and biomechanical instantaneous dynamic elastic modulus (62% of fresh control). A human clinical study using the same method showed 47% viability post-thaw and pre-transplant. Post-transplantation, there was an increase in the knee-specific scores in the patients and plug incorporation in 12 out of 18 patients [10]. As successful as these results may appear, they were not well-received by the surgical community because: (1) directional freezing, as performed, required injection of CPA into the cartilage using fine 20 μm diameter needles, which distort the cartilage matrix upon insertion, (2) ice crystal formation, controlled or uncontrolled, is known to damage the matrix, the cells and cell-matrix junctions, and hence is not desirable, and (3) the reports mentioned that the viability was limited to the superficial layer of the cartilage which is insufficient to maintain the cartilage in the long-term. The 1-year follow-up study also mentioned that the elastic modulus of the cartilage was 40% compromised even before the transplantation and this important property of cartilage was not measured in the 1-year study as the human subjects were still alive and adequate sized biopsies were not possible [10].

## Is vitrification the ultimate answer?

The current collective knowledge regarding successful cryopreservation of cartilage can be categorized into the following facts: (1) articular cartilage needs to remain attached to the underlying bone during preservation for transplantation, (2) the thickness of articular cartilage plays a key role in the success of the cryopreservation protocol, (3) isolated chondrocytes can be cryopreserved using 10% w/w $Me_2SO$ and −1 °C/min cooling rate but this protocol does not work for chondrocytes *in situ*, (4) ice formation within the matrix plays a major role in chondrocyte loss during cryopreservation, and (5) ice formation mechanically disrupts the extracellular matrix which can cause post-thaw/post-transplant failure.

Vitrification is the transition of a solution from the liquid state into a glass-like solid state without forming any crystalline structure, i.e. an amorphous solid. It can be achieved by fast cooling, or by addition of known concentrations of certain solutes, or both. In cryobiology, vitrification involves introduction of high concentrations of cryoprotective agents (CPA) – typically 30–60% w/w CPA – to the tissue [30,33]. If vitreous preservation of cartilage can be achieved, then both the chondrocytes and the matrix can be preserved. Vitrification of pure water is only possible at very low volumes (of the order of cubic microns) and ultrafast cooling rates [14]. The size of the specimen can be a limitation on achieving the desired cooling rates due to heat transfer. Addition of other solutes, such as CPAs, decreases the required cooling rate thus increasing the size of specimen that can be vitrified. At certain high concentrations, dependent on the type of CPA used, vitrification can be obtained regardless of the cooling rate or size of the specimen. Thus, there are three main obstacles to overcome for the successful vitrification of tissues: (1) CPA permeation, (2) CPA toxicity, and (3) CPA vitrifiability (obtaining sufficient concentration to vitrify and not devitrify during warming).

It has been observed that ice formation is correlated with cell damage within the articular cartilage matrix [75,83]. Ice formation alters the collagen matrix and the proteoglycan network by enlarging pores and breaking the protein molecule chains [48,60,109,116]. Fahy et al. (1984) suggested that, upon successful vitrification, the target tissue need not satisfy classical cryopreservation constraints, and can escape both intracellular freezing and the solution effects [30]. This was not adopted until other efforts of classical cryopreservation of cartilage failed, as described in the previous section. Upon successful vitrification, various problems with regards to large tissue and organ cryopreservation can be addressed, including nonuniform cooling and warming rates – which will not be controlled nor as fast as desirable – and ice formation. This was further accentuated by Pegg in an article published in 2001 in which it was suggested that, since vitrification of large tissues (compared to cells) is only possible when CPAs are used in high concentrations, vitrification would be the ultimate answer to the cryopreservation of tissues only when the cytotoxicity of the CPA is properly addressed [81].

There are a considerable number of publications and patents on the application of vitrification for tissue and whole organ preservation including kidney [32], liver slices [29] and blood vessels [55]. Most tissues studied were either vascular or were organ slices, in both cases the CPA equilibration time throughout the tissue could be effectively reduced by the perfusion of the CPA solution or adjusting the tissue slice thickness [56]. The earliest accounts of vitrification of articular cartilage are from Jomha et al. [45,46]. These two studies demonstrated 42% and 33% cell recovery respectively after vitrification using high concentrations of $Me_2SO$. Song et al. achieved ~80% chondrocyte viability (Alamar Blue and calcein-AM fluorescent functional assays) in vitrified rabbit full thickness femoral head cartilage. Using cryosubstitution, it was shown that vitrification, or in other words ice-free cryopreservation, was truly achieved [96]. In another study, scanning electron microscopy of the cartilage samples immersed and fast-cooled in $\geqslant$6 M DMSO solution showed a decrease in the size and total volume of the enlarged pores due to ice formation [48]. Further evidence of the protection of extracellular matrix from ice formation damage was provided by multiphoton fluorescent imaging of cartilage grafts and Raman spectroscopy of heart valve leaflets, concluding that the tissue extracellular matrix received more extensive damage when frozen with a conventional slow-freezing than when vitrified [18,105].

Since the concentrations required for vitrification are generally high, a number of studies have investigated CPA toxicity at high concentrations in cartilage and other tissues providing some valuable information although the data is far from complete. It is clear that CPA toxicity is species and tissue specific; therefore, these results cannot be generalized [5,23,85,88,104,111]. There are few studies investigating the mechanisms of toxicity and the effects

of high concentrations of CPAs [7,13,26,28,32,113]. More recently, a few studies have investigated CPA toxicity specific to articular cartilage with some general trends in CPA toxicity to chondrocytes and CPA interactions developing [6,26,53]. The specifics of cellular toxicity are not clearly defined at this point and methods of mitigating toxicity of specific CPAs are not available; however the general consensus in the field of cryobiology is to expose cells to the CPA at the lowest concentration and temperature for the shortest exposure time possible so the formation of ice is avoided. This method is called liquidus tracking or stepwise loading and cooling.

*Liquidus-tracking and stepwise loading-cooling*

The first notion of step-wise or continuous loading-cooling in the literature was in an article published in *Nature* in 1965 by Farrant, where he suggested that the viability of cells in tissues could be sustained after cooling to temperatures as low as −79 °C, given that the increase in the concentration of electrolytes due to ice exclusion during cooling and freezing was avoided by continuous or step-wise addition of CPAs, such as DMSO or glycerol, to the freezing medium [33]. This approach was motivated by evidence of electrolyte toxicity at high concentrations that occurred during freezing [64]. In fact, increased electrolyte concentration, particularly the sodium ion concentration, is found to have adverse effects on cell viability and homeostasis in various aspects of biopreservation by changing the ionization levels of the phospholipid head groups and their range of electrostatic interactions [44,99]. Nonetheless, even theoretically perfect protocols of cryopreservation did not result in 100% survival of cells all the time, which led Fahy to discuss the evidence of CPA toxicity as a major cause of inefficiencies in protocols of cryopreservation for cells and tissues in an article published in 1986 [27].

In liquidus-tracking, exposing cells to a low concentration of CPA at the lowest temperature possible, i.e. 0 °C, minimizes the concentration- and temperature-dependent toxicity of the CPA. The time-dependent toxicity of the CPA would depend on the rate of the CPA diffusion into the cells. Then the suspension of cells in CPA can be cooled down to the freezing point of the solution to further suppress the temperature-dependent toxic effects. Sequentially, more CPA concentration is introduced to the suspension and time is allowed for equilibration. The steps of concentration increase and temperature decrease continue until a vitrifiable concentration of the CPA is reached within the cells, at which point plunging the suspension of cells into liquid nitrogen vitrifies the system. This approach is feasible with cells in suspension considering that the size of the cells is much smaller compared to tissues (μm vs. mm). The CPA can cross the cell membrane and reach equilibrium within minutes [67,112]. In tissues, however, the time of diffusion and equilibration of CPA can take hours depending on the size and dimensions of the tissue [92] which is far too long for protocols suggested by Farrant in terms CPA cytotoxicity. Elford and Walter were first to practice Farrant's stepwise loading/cooling method using smooth muscle cells [24]. However, the interstitial concentration of the CPA at each step was unknown in that study. In the last of a series of three papers by Pegg [82–84], he describes the first perfectly performed actual liquidus-tracking method. Discs of ovine cartilage with 0.7 mm thickness were vitrified through a 7-step vitrification protocol and the interstitial concentration of the $Me_2SO$ at each step during loading and washing steps was carefully measured. The reported recovery of the chondrocytes, about 57%, was assessed by $^{35}S$ incorporation in a glycosaminoglycan (GAG) functional assay. Later, they reported up to 87% and 70% of control $^{35}S$ incorporation for ovine and human discs, respectively, using a continuous stirring system [106]. It can be debated that functional assays can produce misleading results when performed on cellular systems as these cells can become metabolically more active after cooling stresses, as demonstrated by the work of Jomha et al. [49]. These results were the first positive demonstration of liquidus-tracking application in tissues; however, it suffered from one significant limitation: actual human cartilage with up to 5 mm thickness would require significantly longer times for equilibration of the CPA than cartilage discs with 0.7 mm thickness. The equilibration time would be even longer when the cartilage-on-bone grafts are to be cryopreserved using this method as cartilage on bone has only half of the surface available for CPA diffusion compared to a cartilage disc.

*Single- versus multi-CPA solutions for vitrification*

Using one type of CPA during stepwise cooling for vitrification may be possible in thinner tissues as shown by Pegg but would result in excessive CPA toxicity in larger tissues because of the prolonged exposure to very high concentrations of the CPA during the final stages. This is complicated by the fact that for most CPAs, cytotoxicity increases nonlinearly with concentration [26]. Therefore, to decrease concentration-dependent CPA cytotoxicity during cooling steps, another approach is to use combinations of CPAs each at a lower final concentration so that individually the CPAs are less toxic to the cells but the overall final concentration is sufficient to vitrify [20,31,61]. The idea of cryoprotectant toxicity neutralization using certain amides as structural analogues of cryoprotectants was discussed previously by Fahy [28]. Recently, Jomha et al. showed positive interactions between commonly used CPAs [6,53]. This indicates that a lowered cumulative CPA toxicity occurs in multiple-CPA solutions compared to single-CPA solutions of similar total concentration [53]. The same group showed that multiple-CPA solutions can be beneficial by increasing the glass stability above that of an equivalent molar single-CPA solution [108]. These conclusions were indirectly supported by Brockbank et al. (2010) [17] who recorded good chondrocyte recovery after vitrification of pig articular cartilage using different combinations of $Me_2SO$, formamide and propylene glycol (VS55 and VS83 both loaded at 4 °C followed by cooling to −135 °C at various cooling rates). Cartilage thickness remained an issue as the results showed increasingly lower recovery with thicker cartilage. Again in this study the cartilage had been removed from its bone base prior to vitrification.

## The current status of cartilage vitrification

To address three main obstacles to cartilage cryopreservation, including CPA permeation to prevent ice formation within the matrix, CPA toxicity and CPA vitrifiability, vitrification with multi-CPA solutions using stepwise cooling is perhaps the most viable approach. Various aspects of the cryopreservation process must be considered in order to develop a successful protocol, such as the effect of cartilage thickness on the CPA distribution and loading/equilibration time, inhomogeneity of matrix collagen orientation and proteoglycan distribution and its effect on the CPA permeability throughout the depth of cartilage, and estimation of collective CPA toxicity/solution effects as a function of temperature, concentration and exposure time (dose response).

*Measurement of CPA diffusion in cartilage*

Different measurement methods have been used by researchers to gain an understanding of the diffusion rate of specific CPAs in cartilage and similar tissues. Sharma et al. [92] and Jomha et al. [51] calculated the overall uptake of four commonly used CPAs in cartilage discs by measuring the osmolality of a known amount of phosphate-buffered saline in which the treated cartilage disc had been equilibrated over 24 h. Using a similar approach, Pegg

et al. [106] used high performance liquid chromatography (HPLC) to measure $Me_2SO$ content in discs of cartilage. Wusteman et al. [113] did not directly measure the overall concentration, but used differential scanning calorimetry (DSC) to measure the melting point of the tissue sample after freezing for direct application in their step-cooling protocol. In a few other studies, magnetic resonance imaging (MRI) has been used to evaluate the overall CPA content of the tissue [34,43,80]. Mukherjee et al. (2008) used MRI to obtain total $Me_2SO$ concentration in cartilage dowels [71]. The data acquired in these experiments were either used directly in the design of the stepwise protocols, or were fed to models such as Fick's law of diffusion to calculate the effective diffusion coefficient of the CPA in cartilage for making further predictions. The study by Isbell et al. [43] was the first to demonstrate the possibility of collecting spatially resolved data of the dynamics of CPA diffusion in rat kidney and liver tissues. However, the application of the acquired data was limited to the calculation of an effective diffusion coefficient in the tissue. A recent study by Abazari et al. (2012) was the first to experimentally spatiotemporally resolve the uptake of $Me_2SO$ in cartilage dowels during the course of a 1-h experiment using MRI [3]. The data presented in that study showed that the heterogeneities in cartilage matrix collagen and GAG protein network have minimal effect on the distribution of a nonionic solute such as $Me_2SO$, and that, in full-thickness healthy porcine cartilage, the diffusion of $Me_2SO$ is not significantly hindered due to matrix orientation and density across the thickness, and that the diffusion is abruptly impeded at the bone-cartilage interface, as previously suggested [78].

*Modeling CPA transport in cartilage*

A nonuniform distribution of CPA due to the thickness produces a subsequent nonuniform pattern of damage, so that the chondrocytes may survive in some regions while experiencing more damage in other regions. This makes it even more difficult to analyze the CPA toxicity effects during loading. Therefore, minimization of the exposure time of the tissue to the CPA is a necessity for minimal damage to the cells in the tissue. In a CPA-loading protocol, steps must be designed to minimize the exposure time at each temperature. Therefore, knowledge of CPA diffusion in cartilage, by measurement or by calculation, is required for the design of effective and efficient CPA-loading protocols. However, modeling efforts for predicting CPA diffusion in tissues such as articular cartilage have been few and limited until recently. Muldrew et al. used Fick's law to calculate the diffusion coefficient of the $Me_2SO$ in cartilage for further predicting the overall $Me_2SO$ uptake in cartilage over time [76]. Maxwell–Stefan transport equations were used by Xu and Cui (2003) in modeling the co-transport of multiple solutes in a porous media for applications in tissues such as cartilage [114] – Maxwell–Stefan equations are a more sophisticated set of equations from which Fick's law can be derived using some simplifying assumptions including an ideal-dilute assumption for solutes. Two different studies were published in 2008 by Zhang and Pegg [115] and Mukherjee et al. [71] on modeling CPA diffusion in cartilage. Mukherjee et al. used Fick's law of diffusion to predict the spatial and temporal distribution of the CPA in cartilage. That information was further used to design hypothetical stepwise cooling protocols and predict the chondrocyte volume response to CPA loading. Lawson et al. used the same approach to simulate stepwise loading and removal of CPA from tissues [62]. These predictions are of high practical importance for designing and optimizing liquidus-tracking or stepwise loading-cooling steps. Whether or not Fick's law is capable of making accurate predictions is another important question. To answer this question, Zhang and Pegg [115] utilized the triphasic model of cartilage by Lai et al. [59], developed in the biomechanical engineering field, to describe the movement of the CPA in cartilage. As novel as the study by Zhang and Pegg was, some of the assumptions were insufficient for the specific case of vitrification solutions, and basically reduced the model to Fick's law. For example, the assumption of ideal and dilute solutions for vitrifying concentrations of the CPA was insufficient. Also, osmotic movement of the interstitial fluid was ignored in the analysis. In addition, in part due to lack of appropriate data, no values were reported for the transport parameters of the model other than the diffusion coefficient of the CPA. Therefore, the final conclusion of the study was that there were no essential differences between the biomechanical model and Fick's law in calculating transport in cartilage. Abazari et al. (2009) took the biomechanical model one step further by including non-ideal solution thermodynamics and by incorporating appropriate data (not only measured overall concentration in the cartilage disk but also its weight change) and were able to calculate transport parameters of the biomechanical model [2]. Later, they incorporated the natural collagen and GAG network inhomogeneities into the model to calculate a more realistic set of parameters, and show that during CPA loading, cartilage undergoes a shrinking stress resulting from osmotic water loss from within the tissue toward the surrounding solution [4]. A recent study by the same group, comparing spatially and temporally resolved measurements with Fick's law and the new biomechanical model, showed that the CPA distribution can be significantly underestimated when using Fick's law [3]. Such underestimation can result in longer than necessary CPA exposure of the chondrocytes within the matrix hence increasing the time-dependent toxicity of the CPA.

*Dehydration and osmotic stresses during CPA loading*

During CPA loading to cartilage from a concentrated surrounding solution, there is an osmotic water flow to and from the cartilage when exposed to solutions of different osmolalities which causes shrinking and swelling of articular cartilage during the CPA loading (and removal) which was not accounted for in the cryobiology literature before the works of Abazari et al. [2,4]. In the context of biomechanical engineering, however, this water movement is known and included in the triphasic model of cartilage under mechanical or osmotic stress [39,59,70]. Cartilage exhibits osmotic behavior similar to biological cells when exposed to different tonic environments: it swells and shrinks when exposed to hypo- and hypertonic solutions. The osmotic properties of cartilage are due to the presence of specific proteins within the cartilage matrix called proteoglycans. It is known that these osmotic properties contribute to the weight-bearing properties of articular cartilage by partially balancing the mechanical stress [69]. When cartilage is exposed to concentrated CPA solutions, it shrinks and dehydrates due to osmotically-driven water movement from the matrix to the solution. The extent of the shrinkage and the resultant stress–strain in the tissue matrix and effects on the chondrocytes may be important issues as described by Abazari et al. [4]. Also, after the diffusion of the CPA into the interstitial fluid, the tissue gains back the volume and swells. This shrink-swell behavior can be repeated a few times when cartilage is treated in a multistep loading protocol. In the biomechanical engineering literature, the adverse effect of cyclic mechanical stress and strain in the tissue matrix on the chondrocytes has been demonstrated [57]. Also, dehydration of cartilage and concentration of the salt ions in the interstitial fluid and the diffusion of the CPA into the cartilage change the osmotic environment of the chondrocytes [4]. The osmotic stresses on the chondrocytes under CPA loading protocol conditions have generally not been considered important in the field of cryobiology. It has been observed that osmotic dehydration causes a significant decrease in cellular activity [91]. Simulation results for stress–strain in the cartilage matrix

during a hypothetical CPA-loading protocol have shown that the middle and deep cartilage may experience a significant mechanical stress due to outward osmotic water flow, which would also influence the interstitial ionic environment, resulting in an hyperosmotic environment for chondrocytes [4]. Such modeling results can provide an explanation for some unexpected outcomes seen in other studies, where in transplantation follow-up studies, only chondrocytes in the superficial layer survived while the middle and deep layers were observed to be acellular [72,74].

## Summary of the challenges and suggested approaches

Both the cellular system and the ultrastructure of the cartilage matrix are required to be efficiently preserved for any cryopreserved-cartilage transplant to be successful in the long term. To achieve this, vitrification is the approach that has been successful. For vitrification of cartilage, where no vascular system exists to facilitate the CPA transport into deep cartilage, the major hurdle is CPA permeation into thick cartilage, during which the chondrocytes are exposed to potential CPA cytotoxic effects. The eventual answer to the thickness problem requires a combination of the following approaches: (1) stepwise loading-cooling, whereby decreasing the cartilage-bath system temperature to reduce the cytotoxic effects is in concert with the increase in CPA concentration as the CPA is gradually introduced, and (2) use of multiple-CPA solutions instead of single-CPA solutions. It must be noted that an adverse effect of the liquidus-tracking method is that, since the CPA diffusion rate has an Arrhenius temperature dependence, lowering the temperature also slows down the rate of CPA transport within the tissue. For example, the Fickian diffusion coefficient for $Me_2SO$ decreases by 25% going from 0 °C to −10 °C [51]. This temperature dependence is even more significant for some other common CPAs such as glycerol and propylene glycol, which decrease about 50% within the same temperature range [51]. This means that longer diffusion times are needed to reach the same desired concentration, which also means longer exposure of the chondrocytes to the CPA, hence higher toxicity. Additional information that is important to improve the success of vitrification protocols includes: (3) dose-dependence of CPA cytotoxicity, which is required to be clearly defined as a function of temperature, concentration and exposure time, and (4) modeling, which will facilitate the design of loading protocols and will greatly reduce the number of trial and error experiments.

Recently, successful vitrification of intact human articular cartilage on its bone base has been achieved by Jomha et al. [52] by incorporation of all the aforementioned elements. Early work with single-solution high concentrations of $Me_2SO$ (Jomha et al. [45,46]) resulted in moderate cell recovery after vitrification and was followed by investigations into CPA permeation kinetics [2,51,92], CPA toxicity kinetics [6,26,53] and CPA vitrifiability [108]. The data gathered from these studies, combined with the ability to calculate freezing points of multi-CPA solutions [25,86], was incorporated into a stepwise vitrification protocol where four CPAs were added at progressively lowered temperatures until 6.5 M concentration was reached [52]. The tissue consisted of 10 mm diameter osteochondral dowels (cartilage on the bone) as well as larger fragments approximating 12.5 $cm^2$ and was obtained from knee replacement surgeries as well as normal articular cartilage from deceased donors. The tissue was vitrified in liquid nitrogen for up to 3 months. Cell recovery was over 75% on 18 different samples from 10 different human knee replacement surgery donors with similar results from large fragments, normal cartilage from deceased donors and after storage for 3 months in one sample [52]. Cell viability was determined by membrane integrity stains as well as a mitochondrial assay and a functional assay consisting of pellet culture of the cells followed by staining for cartilage specific sulfated proteoglycans and collagen type II [52].

This paper has presented a review of some of the important understanding that has been gained in the area of articular cartilage cryopreservation, from early work on the cryopreservation of isolated chondrocytes in the 1950s and 1960s through to recent reports of vitrification of articular cartilage of various species both removed from the bone and intact with its bone base.

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