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ALGINATE ENCAPSULATION TO ENHANCE BIOPRESERVATION SCOPE AND SUCCESS: A MULTIDISCIPLINARY REVIEW OF CURRENT IDEAS AND APPLICATIONS IN CRYOPRESERVATION AND NON-FREEZING STORAGE

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Abstract

BACKGROUND: The development of encapsulation technologies has played an important role in improving cryopreservation outcomes for many cell and tissue types over the past 20 years. Alginate encapsulation cryopreservation (AECryo) has been incorporated into a range of applications in biotechnology, species conservation and clinical therapies, using cells from many different phyla, including higher plants, animal and human cells. This review describes the background to the origins of AECryo, the development of AECryo in higher plant tissues, broadening to current applications in algal conservation, the roles for AECryo in preserving phytodiversity, fungal species and in animal and human cells. **OBJECTIVE:** The main aims are to provide information resources on AECryo in different areas of biology and to stimulate new ideas for wider applications and future improvement. The translation of this useful biopreservation strategy into new opportunities for cell cryopreservation and storage at non-freezing temperatures are also discussed.

Keywords: alginate, encapsulation, cryopreservation, plant tissues, algae, fungal species, mammalian and human tissues and cells, hypothermic storage, phytodiversity, biotechnology, regenerative medicine.

INTRODUCTION

Over the past two decades, applications of cryopreservation have proceeded hand in hand with developments of other technologies, which have been designed to address problems in biotechnology, cell therapy and species conservation. Cell encapsulation in alginate is one such technology, which can facilitate several critical steps within cryopreservation, such as controlled handling of sensitive cells or tissue, exposure to cryoprotectant agents, and enhanced

stability of the cryopreserved products (58, 92, 127, 182). This review is aimed at providing current information on alginate encapsulated cryopreservation including the nature of the alginate biopolymer and challenges with its use in regulated cryopreservation practices, a history of alginate encapsulation cryopreservation (AECryo) and our understanding of the chemical and physical advantages of applying alginate technologies, and the results achieved so far across a range of different species. It is presented under the following headings:

1. ALGINATE HYDROGEL AS A SAFE, CONTROLLED POLYMER FOR CRYOPRESERVATION.

2. ALGINATE ENCAPSULATION IN PLANT CRYOPRESERVATION – HISTORICAL DEVELOPMENT OF AECryo CONCEPTS

3. ALGINATE ENCAPSULATION FOR THE CRYOPRESERVATION OF ALGAE

4. ALGINATE-BASED CRYOBIOLOGY FOR PHYTODIVERSITY CRYOPRESERVATION

5. ALGINATE ENCAPSULATION FOR FUNGAL CRYO-STORAGE

6. ALGINATE ENCAPSULATION CRYOPRESERVATION APPLIED TO ANIMAL AND HUMAN CELLS AND STEM CELLS

7. ALGINATE ENCAPSULATION FOR STORAGE OF CELLS AT NON-FREEZNG AND POSITIVE TEMPERATURES

1. ALGINATE HYDROGEL AS A SAFE, CONTROLLED POLYMER FOR CRYOPRESERVATION.

Alginates comprise a range of biopolymers, which can be extracted at commercial scales from brown algal species including *Lessonia nigrescens* and *L. trabeculata* and have been widely used in the food and pharmaceutical industries for many years (123, 162, 183) as bulking or stabilising agents due to their low toxicity. They are polysaccharide copolymers consisting of linear 1,4 linked residues of β-D-mannuronic acid (M) and α-D-guluronic acid (G) arranged in blocks of consecutive M or G residues or mixed M and G residues (123, 162). Particular compositions are dictated by the species of origin and the environmental growth conditions, given that the algae are widely distributed around different continental coastlines (13, 163); for example, G composition can vary between 30 – 70%. These differences may be of relevance when selecting alginates for encapsulation cryopreservation since differences in M to G ratios can affect physical characteristics such as viscosity of the solubilised alginate or stiffness of the polymerised form (154, 183).

Sodium alginate is provided as a commercial powder, which can be solubilised in aqueous solutions by extensive mixing. The polymerisation to a gel format suitable for encapsulation can be driven by addition of multivalent cations (such as Ca^{2+}, Ba^{2+}, Fe^{3+}) which interact with carboxyl groups of the carbohydrate residues (123, 182), and due to toxicity issues surrounding metal ion exposure, Ca^{2+} has been most commonly used for encapsulation / cryopreservation. The M:G ratios of a particular alginate fraction, the viscosity of the solubilised form (which is related to concentration) and the cation employed in polymerisation were found to be the most important factors in determining the stability of the capsules produced after polymerisation (33, 154). In general, alginate has been found to be a non-toxic, biocompatible polymer with a wide range of cell based applications (75) and thus is a very useful candidate for encapsulation cryopreservation. The polymerisation process can be reversed by exposure to chelating agents such as EDTA (53), although this is not an often reported step in most encapsulation / cryopreservation strategies. Alginate encapsulated cells may also be subject to capsule instabilities when exposed for long durations to physiological monovalent cations which exchange with the divalent cations [for example after transplantation of cryopreserved encapsulated cells (19)] although this is not specifically related to the cryopreservation process.

An important point when applying alginate encapsulation / cryopreservation is the potential contaminants in the crude commercial polymer, which can comprise inorganic and organic materials such as bacteria, fungi, animal waste and others encountered within the marine environment (155, 183). For example, spores of gram positive bacteria have been found in commercial alginate fractions (182). Extraction processes to yield commercial polymer employ

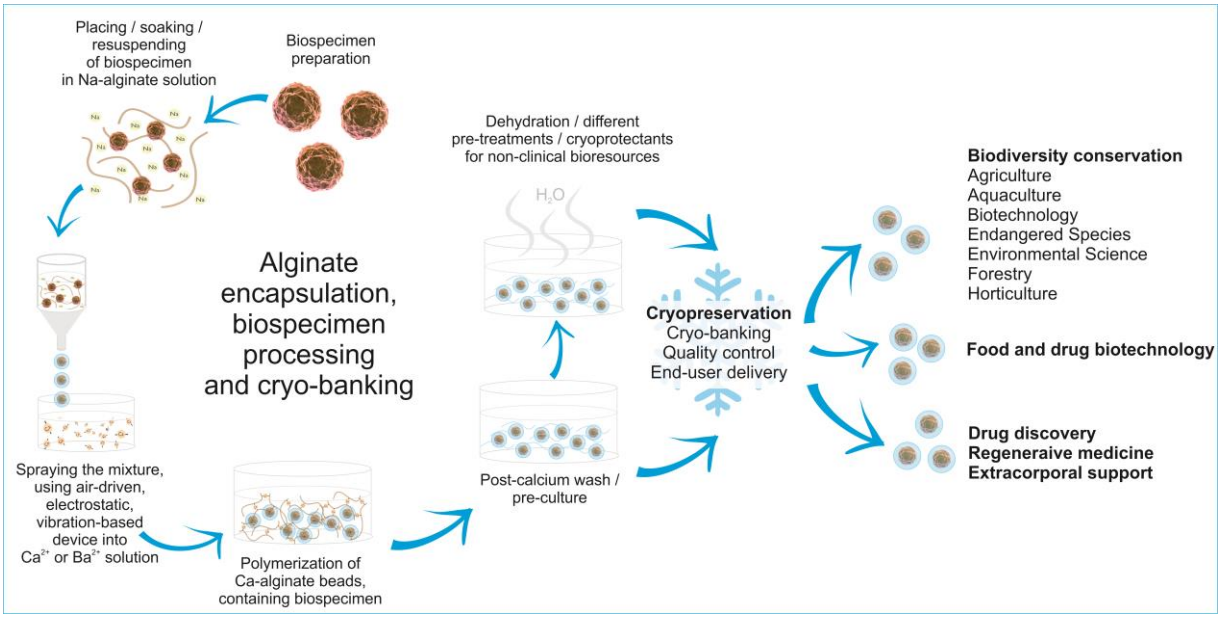

Figure 1. Schematic generic representation of the typical process for alginate encapsulation, biospecimen processing and cryo-banking. After preparation, the biospecimen is soaked in sodium (Na) alginate solution and sprayed (or transferred) to polymerization solution containing multivalent cations (Ca^{2+}, Ba^{2+}, Fe^{3+}). Following polymerization and washing steps, encapsulated biospecimens are ready for further processing and analysis. Cryopreservation of encapsulated samples may be conducted either directly by saturation with the cryoprotective solution and programmed freezing or rapid cooling, and progressed through an additional step of dehydration, preincubation or pretreatment.

chelation steps and washes in various organic solutions are standardised by adherence to American Society for Testing and Materials (ASTM) protocols (41), but these may not guarantee complete removal of biological contaminants.

2. ALGINATE ENCAPSULATION IN PLANT CRYOPRESERVATION – HISTORICAL DEVELOPMENT OF AECryo CONCEPTS

Immobilization and encapsulation technologies have been used over several decades in plant agriculture, biotechnology, forestry, horticulture and environmental sciences, thus reflecting their diverse applications across multidisciplinary sectors (85, 118, 146). Plant technologies have in fact driven the conceptualisation and practical development of AECryo several years before their applications in animal or human cell cryo-storage.

Alginate encapsulation in plant biotechnology: cellular and developmental aspects

The use of alginate bead encapsulation as a cryoprotective strategy is based on artificial seed technology and was first explored by Fabre and Dereuddre for potato shoot meristem cryostorage (47). The concept of making artificial seeds by encapsulating plant somatic embryos in calcium alginate was pioneered in the 1970s for clonally propagated species and elite genotypes (118). Dereuddre et al. (39) adapted the approach to cryopreserve alginate encapsulated somatic embryos of *Daucus carota* achieving > 90% survival after ultra-rapid cooling in liquid nitrogen (LN). Encapsulation has the advantage of allowing the direct transfer of clonal propagules to the glasshouse or field and it provides a convenient carrier for growth regulators, nutrients and antimicrobials. Incorporation of bioactive agents directly into the gel matrix supports the growth and development of somatic embryos, meristems, nodal segments and shoot cuttings and encapsulated propagules can be dried and stored until required for use. Synthetic seed technology has potential utility in horticulture (20) and the sustainable forest products sector (31) although commercial scale-up of 'Synseeds' is elusive in the conifer forestry (17).

The exploitation of alginate encapsulation in plant cryopreservation is also preceded by its

use in the natural products industries in which polymer matrices are used for the transformation and production of secondary metabolites from plant enzymes, cells, tissues and organs (21). Immobilization of plant cells in gels has advantages for large-scale culture in bioreactors as the process supports continuous operations and separates biomass from the medium. The productive life of immobilized cultures can be extended and encapsulation stimulates secondary metabolite production in some cell lines (137). Whilst alginate immobilization has proven its utility in plant biotechnology and biopreservation, little is known about how encapsulation influences plant cell metabolism, growth and development. Some clues can be found in the early plant protoplast literature, which reports that ageing in isolated protoplasts can be reduced by encapsulation. Schnabl et al. found that the immobilization of *Vicia faba* protoplasts in calcium alginate delayed the production of cell ageing biomarkers (133). Immobilization in the alginate matrix inhibited indicators (biomarkers) of free radical mediated lipid peroxidation, delayed pigment degradation and reduced protease activity. It was presumed that Ca^{2+} and alginate retarded degradation reactions, protected cell membranes and increased protoplast stability. Schnabl et al. inferred that when cells die in the gel matrix the proteolytic enzymes released diffuse slowly and are inhibited from degrading neighbouring viable cells and proposed alginate encapsulation as a protoplast storage method (133). Schlangstedt et al. (132) found that encapsulation stimulated the development of *Beta vulgaris* protoplasts in the presence of a nurse culture and suggested that alginate gels provide a 'gentle environment' that protects cells against mechanical damage.

A novel approach to plant cryopreservation: encapsulation/dehydration

Cryopreservation using alginate bead encapsulation was initially developed for potato by Fabre and Dereuddre (47), the motivation being that shoot meristems derived from *in vitro* cultures proved persistently difficult to cryopreserve (18, 62). The novel idea of adapting artificial seed technology for cryostorage was tested as an alternative to using cryoprotectants combined with controlled rate cooling. In brief, the process involves: i) encapsulating shoot meristems in a calcium-alginate matrix; (ii) osmotic dehydration of the bead/meristems in sucrose solutions; (iii) evaporative desiccation in a sterile air flow, or over silica gel or drying beads; (iv) direct plunging into LN; (v) rewarming at ambient temperature, usually in a laminar airflow cabinet; (vi) rehydration of the beads in liquid medium to remove sucrose; and (vii) transfer to recovery medium. Each step of the protocol is optimized for specific species and genotypes. Agents that support recovery and regrowth can be added to the alginate as demonstrated by the increased post-cryopreservation regeneration of yam shoot meristems in alginate beads loaded with melatonin (165). Encapsulation/dehydration has the advantage of being a 'low tech' cryopreservation protocol as it is amenable to laboratories that do not have access to controlled rate cooling equipment. However, good logistics and forward planning are required as the protocol involves multiple steps undertaken over 1-2 days and the method can be labour intensive when processing large numbers of samples. To assure the reproducibility of the air desiccation step silica gel evaporation is advised for laboratories operating in unregulated ambient environments (143). Several modifications have been made to the original encapsulation/dehydration protocol to improve the tolerance of different plant species to dehydration and desiccation (105, 167, 171).

Encapsulation/dehydration has been applied to over 70 plant species (51) and is particularly suitable for the cryopreservation of organized tissues such as shoot tips, somatic embryos and microspore embryos (38, 44, 59, 119, 166). Encapsulation-based methods provide an alternative method for the cryopreservation of germplasm from plant species and genotypes that produce storage recalcitrant seeds and/or that do not respond to controlled rate cooling and for which chemical cryoprotectants may be toxic (9, 103). For certain sectors (e.g., horticulture, clonal forestry and agroforestry) alginate encapsulation has the advantage of combining artificial seed technology (59, 118) with *in vitro* conservation (31). Encapsulation/dehydration has been used for the cryopreservation of a broad range of plant taxa (51) including cryptogams (87) and the method has also been adapted for the *ex situ* conservation of rare species (50, 88). Encapsulation/dehydration protocols have been validated for the cryopreservation of clonal crop germplasm maintained in international genebanks (120, 122).

Although alginate-based cryopreservation was initially pioneered for tropical plants that are recalcitrant to other cryopreservation methods (45), desiccation-sensitive germplasm can still succumb to stress. Understanding the physiological basis of plant cryopreservation success and failure will be important for the improvement of cryostorage outcomes in the future (9, 56). This is also the case for temperate plants that have complex seasonal life cycles impacted by environmental cues (9, 49).

Figure 2 demonstrates recovery responses for the initial survival of apical meristems (A) and lateral shoot regrowth (B) of *Picea sitchensis* (Sitka spruce) following alginate encapsulation/dehydration, evaporative desiccation and exposure to LN. After cryostorage some regrowth was observed (B) although necrosis occurred (C, D) following the initial recovery of the lateral meristem, which was not sustained in the longer-term. This study (49) addressed the importance of understanding the complex relationship between stress-induced dormancy and cryoinjury in the sustained recovery of encapsulated germplasm derived from a recalcitrant, temperate tree species which has a complex life cycle. The decline in viability over extended recovery times observed in Sitka spruce shoots shares commonality with the hypothesis of delayed onset, cryopreservation induced cell death which is attributed to apoptosis in the field of biomedical preservation (7).

Enhancing the utility of alginate-based plant cryopreservation using encapsulation-vitrification

Matsumoto and Sakai (94) and Sakai et al. (127) modified the original encapsulation/dehydration method developed by Fabre and Dereuddre (47) and Dereuddre et al. (39) by combining the alginate bead dehydration

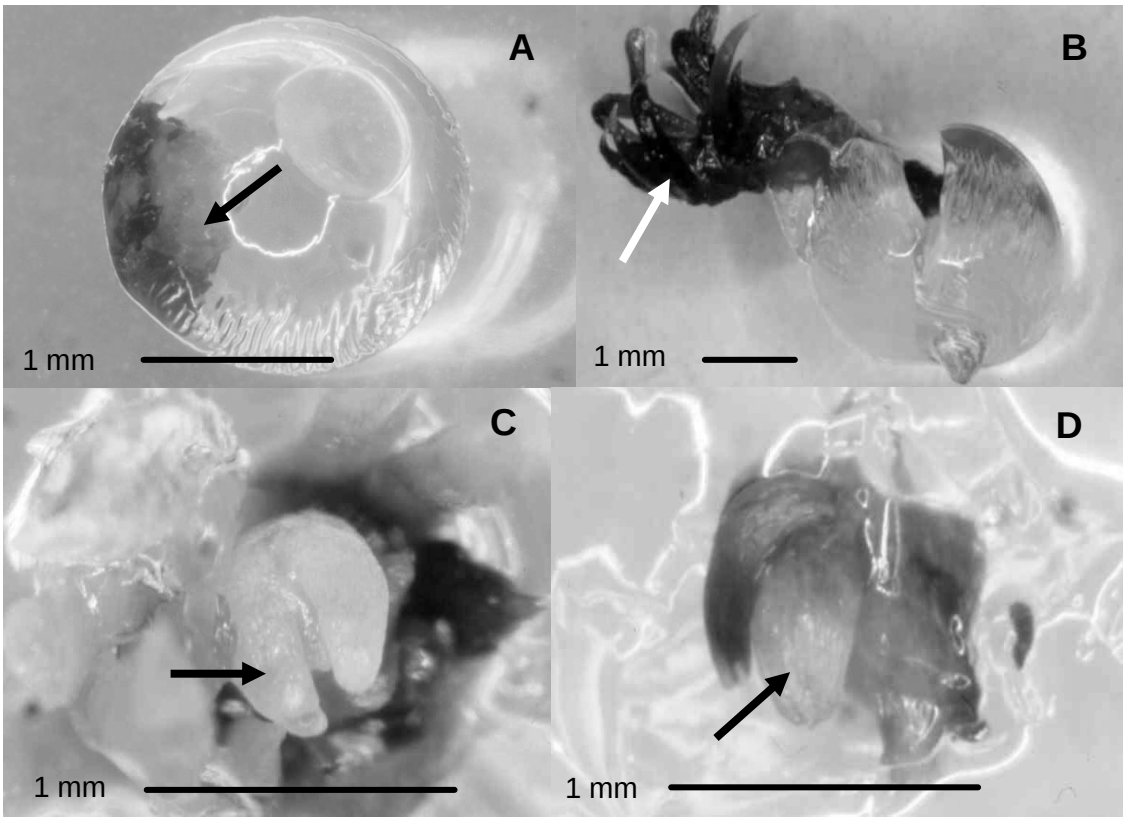

Figure 2. *Picea sitchensis (Sitka spruce)* meristem apices recovering after cryogenic treatments. **(A)** green apical tissue (E+S+D = Encapsulation + Sucrose + Desiccation); **(B)** lateral meristem emerging from bead (E+S); **(C)** lateral meristematic dome, green primordia with black bud scales (E+S+D+LN); **(D)** initial lateral meristem regrowth followed by necrosis (E+S+D+LN). Meristem apices were assessed after 28 (A-C) and 48 days (D). Treatments: alginate encapsulation (E); 0.75 M sucrose for 18 h (E+S); 4 h air desiccation (E+S+D); LN (E+S+D+LN). Reproduced with permission from CryoLetters [Gale et al. (9)].

step with osmoprotection in a solution of glycerol and sucrose which enhances resilience to osmotic dehydration and evaporative desiccation treatments. This approach was later developed as the 'encapsulation-vitrification' protocol by Sakai and colleagues (128) who applied it to shoot meristems from species sensitive to dehydration and desiccation. Encapsulation-vitrification combines alginate encapsulation with the application of cryoprotective additives [dimethylsulphoxide (DMSO), glycerol, sucrose, and ethylene glycol] that comprise Plant Vitrification Solution Number 2 (PVS2). The basic procedure involves: (i) pregrowth of donor shoots with sucrose; (ii) alginate encapsulation of meristems in which sucrose, or a mixture of glycerol and sucrose are preloaded; (iii) cryoprotection with PVS2; (iv) direct immersion in LN; (v) rewarming of the alginate encapsulated meristems at 35-45°C; (vi) removal of PVS2 and replacement with a sucrose unloading solution. Each step requires optimizing for species and sensitive genotypes. By reducing or removing dehydration and desiccation steps, encapsulation-based cryopreservation becomes more amenable to desiccation-intolerant plant tissues (127, 128). The encapsulation-vitrification protocol also allows the efficient processing of large numbers of plant meristems by eliminating lengthy dehydration and desiccation treatments. The method has been applied across diverse plant taxa (44, 128) and is particularly useful for species that are recalcitrant to traditional cryopreservation methods (103). Adaptations of the basic encapsulation/vitrification protocol combined with passive, controlled rate cooling have been used to cryopreserve plant cell cultures (72).

Alginate-based cryoplate methods

Yamamoto and colleagues (117, 176, 177) developed an aluminium plate (V-cryoplate) method for the cryopreservation of plant tissues by modifying the PVS2 encapsulation/vitrification approach of Sakai et al., (128). Two types of cryoplates, with different dimensions, each with 10 wells to accommodate shoot meristems were designed to fit into 2 ml cryotubes. In brief, the method involves: (i) pouring sodium alginate supplemented with sucrose into the wells; (ii) placing precultured shoot tips into each well, ensuring that they are fully submersed in the alginate; (iii) dispensing $CaCl_2$ supplemented with sucrose, drop-wise on the shoot-tips/alginate solution and waiting to achieve complete polymerization; (iv) removal of excess $CaCl_2$ from the wells; (v) loading the cryoplate and the alginate/shoot tip wells with a glycerol/sucrose osmoprotectant loading solution; (vi) removal of the loading solution and replacement with PVS2 cryoprotectant; (vii) transfer of cryoplates to cryotubes and direct immersion in LN; (viii) retrieval of the cryotubes from LN; (ix) immersing the cryoplates in sucrose unloading solution and rewarming at room temperature; and (x) transfer of the shoot-tips to culture medium for recovery. Each step of the protocol is optimized for specific species and genotypes. The V-cryoplate modification was implemented to improve the standardization of the encapsulation/vitrification protocol for large-scale cryopreservation in plant genebanks (117, 176). This method has also been adapted for encapsulation/dehydration using silica gel and drying beads for the cryopreservation of protocorms of *Arundina graminifolia* (30).

3. ALGINATE ENCAPSULATION FOR THE CRYOPRESERVATION OF ALGAE

Alginate is produced by all brown algae which can comprise up to 40% alginate (dry weight basis); commercial alginates are mainly extracted from the genus *Laminaria* (100). The immobilization of algae in alginate has well-established applications in biotechnological metabolite production (146) as well as environmental pollution and aquatic research. Alginate gels used in combination with silica matrices have been tested as biocompatible structures for creating portable microcosms comprising different assemblages of organisms, including microalgae (111). The advantages of immobilizing algal cells in alginate are null toxicity, protection from deleterious physical and chemical changes, gel permeability, convenient addition and release of metabolites and visual transparency (100). Calcium alginate encapsulation has been used for the medium-term storage of microalgae in culture collections (29).

Development of alginate-based cryostorage methods in algal culture collections

Cryopreservation is used for the long-term stabilization of algal culture collections (35) for which controlled rate cooling in combination with cryoprotective additives is the usual method

of choice (160). Alginate encapsulation has been pioneered as an alternative approach especially for species and strains that are recalcitrant to traditional cryostorage protocols (55, 63). Hirata et al. first tested encapsulation/dehydration for the cryopreservation of *Dunaliella tertiolecta* and subsequently used the method to cryopreserve six marine microalgae, one freshwater microalga and four strains of freshwater cyanobacteria (63). The protocol was adapted from that developed for plants and, in brief, involves: (i) encapsulating microalgae in sodium alginate polymerized with $CaCl_2$; (ii) osmotically dehydrating the cells with sucrose at an optimal level and duration of exposure; (iii) bead desiccation in a cultivation chamber to a dehydrated bead weight of 30% of the initial fresh weight; (iv) direct immersion in LN; (v) rewarming at 37˚C in a water-bath; and (vi) post-storage cultivation using standard methods and optimal light regimes.

Vigneron et al. (1997) applied encapsulation/dehydration for cryopreservation of gametophytes of the marine alga *Laminaria digitata* (168). The encapsulated gametophytic cells were pretreated for 6 h in liquid medium containing 0.3 - 0.5 M sucrose to within the range of 70 - 90% (fresh weight) followed by desiccation in a sterile laminar air flow. Cooling was undertaken in two steps, the first involved slow cooling from 19˚C to -40˚C and direct immersion into LN. The beads were rewarmed in a water-bath at 40˚C and recovery time was 2 weeks (168). Encapsulation/dehydration with or without two-step cooling was initially applied with some success for the cryopreservation of *E. gracilis* (34). This method was subsequently explored by Harding et al. (55, 58) for the cryopreservation of a wider taxonomic range of microalgae that were unresponsive to controlled cooling protocols. The general procedure for the encapsulation-based cryopreservation of algae is similar to that applied to plants and has the advantage of being amenable to culture collections that do not have access to cooling equipment. Rewarming can be conveniently undertaken at ambient temperatures in a laminar air flow hood followed by rehydration of the beads in liquid medium to remove sucrose, before transfer to recovery medium. The dissolution of alginate beads using the detergent sodium hexametaphosphate may be required so that cells and organisms can be released, recover and replicate (81). Na-alginate does not dissolve effectively in sea or salt water and caution is required during intended bead dissolution as sodium hexametaphosphate is a powerful antisalination agent which can interfere with seawater-based media used to culture marine organisms (100). The potential of using alginate/encapsulation for the cryopreservation of recalcitrant algae has been tested for *Porphyridium aerugineum* (2). The cryopreservation of the marine diatoms *Nitzschia closterium f. minutissima* and *Chaetoceros muelleri* using encapsulation/vitrification has been investigated by Zhang et al. (181) who demonstrated that optimal PVS2 loading of alginate beads supported the highest post-cryopreservation viability of ~74% for *Nitzschia closterium.* Kumari et al. (76) improved recovery of *Oocystis* spp. and *Anabaena* spp. using an open encapsulation/PVS2 vitrification system in which samples are directly exposed to LN rather than being enclosed in a cryovial. The addition of antioxidants 2-mercaptoethanol and glutathione on post-warming improved the viability of algae in both open and closed systems.

Validation and quality management of encapsulation-based protocols in algal cryobanks

The alginate/encapsulation protocol has been successfully applied by European culture collections for the cryopreservation of a diverse taxonomic range of microalgae (36, 43, 55, 58, 81).

Figure 3 demonstrates the application of alginate encapsulation for the cryopreservation of microalgae held by the culture collection of the Sammlung Von Algenkulturen, Universitat Gottingen (SAG), Germany. The duration of exposure to evaporative air desiccation, the use of silica gel and the dark/light conditions applied in the initial post-storage recovery period was found to be critical for the survival of some species (58).

The large-scale cryopreservation of diverse algal taxa in culture collections (Fig. 3) has been supported by international networking and European infrastructures projects that have focused on cryobank methods validation and quality management (10, 36). These resulted in alginate-based methods being validated and deployed across a range of taxa maintained in European algal culture collections (43, 58, 81). The verification of identity and genetic stability in cultures recovered from algae cryopreserved

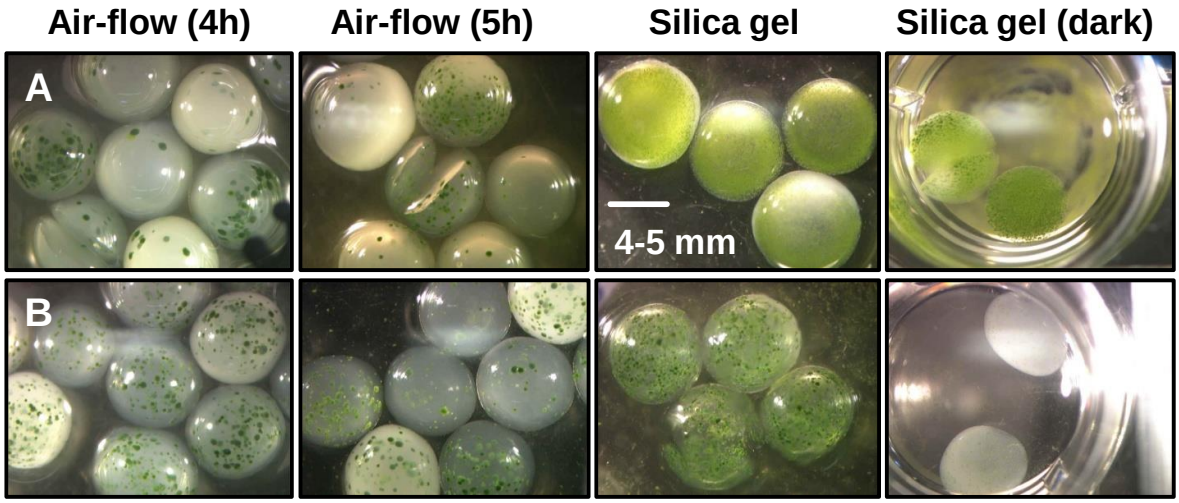

Figure 3. Micrographs representative of typical regrowth responses from alginate encapsulated cryopreserved SAG strains of microalgae: (A) *Parachlorella kessleri* SAG 211-11g and (B) *Chlorella trebouxioides* SAG 3.95 following encapsulation, dehydration and exposure to different desiccation treatments. Reproduced with permission from CryoLetters [Harding et al. (58). SAG = Sammlung Von Algenkulturen, Universitat, Gottingen, Germany.

using both traditional and alginate-based protocols also has been undertaken as a quality assurance measure (57, 101).

As culture collections continue to underpin algal biotechnologies, environmental science and research it will become increasingly important to develop robust quality management systems for algal cryobanks. Bui et al. (23) caution that although the mechanisms underlying algal cryopreservation are generally understood the impacts of the many technical variations in storage protocols reported in the literature are not always elaborated, making it difficult to optimize and standardise protocol variables to maximise post-storage recovery (Fig. 3). To address this issue a new approach to biopreservation quality management the Standard PRE-analytical Code (SPREC) which was first developed for clinical biobanks (14) has been adapted for algal culture collections. A prototype SPREC which systematically annotates and documents the variables to which algal cultures are exposed during culture and cryopreservation has been constructed for microalgae (10). The code includes all possible permutations of cryopreservation variables including those incorporating alginate encapsulation; the algal SPREC has the flexibility to be expanded as new protocols and modifications to existing protocols are developed. Bui et al. (23) suggest that the examination of key variables in algal cryopreservation is necessary to make further improvements possible and that a more comprehensive investigation of these variables is of interest. This concept concurs with the recent development of new reporting procedures and standards for biodiversity biobanks and collections which involves sample reporting and biopreservation process chain variable annotation (11).

4. ALGINATE-BASED CRYOBIOLOGY FOR PHYTODIVERSITY CRYOPRESERVATION

The cryoprotective modality of alginate cryopreservation as applied to cyanobacteria, algae and plants is vitrification, various treatment combinations can be applied to create the glassy state including: evaporative desiccation using air or chemical desiccants such as silica gel; osmotic dehydration treatments (e.g., sugars, alcohols, polyols) combined with alginate bead encapsulation and evaporative desiccation (encapsulation/dehydration) or the applications of chemical cryoprotectants combined with alginate bead encapsulation (encapsulation-vitrification). Vitrification treatments impose extreme biophysical and chemical stress that can be detrimental to plant, algal and cyanobacterial cells, therefore tissues of less tolerant plant species are usually acclimated before they are cryopreserved. Two acclimation strategies are used: 1) exposure to

pregrowth additives (e.g. sucrose, mannitol, sorbitol) that simulate seasonally-induced cold acclimation in dormant tissues (9, 122, 141) or 2) exposure to cold hardening treatments (121). Cold hardening was found to improve the recovery of pear shoot meristems cryopreserved using encapsulation/dehydration (39, 136) and the same approach has been successfully incorporated into alginate-based protocols for a wide range of plant species (119). To achieve the best possible post-storage recovery the optimization of all stages of encapsulation protocols may be necessary for certain species and genotypes (9, 55, 114). Usually alginate encapsulated cells and tissues are cooled at ultra-high rates by direct exposure to LN, although in the case of microalgae controlled, two-step cooling can be combined with encapsulation and chemical cryoprotection (9, 35, 55).

Alginate vitrification, critical factors and Tgs

Cryopreservation involving encapsulation/ dehydration and encapsulation/vitrification involves a change in state from a liquid to a glass, which is defined by a characteristic glass transition temperature (Tg). As glasses are metastable the stabilization of the thermal properties of alginate is crucial for post-storage recovery (9). Differential scanning calorimetry (DSC) elucidates the physical thermal parameters (freezing, melting, Tgs) that are critical for the survival of cryopreserved encapsulated tissues thus thermal analyses can be used to optimize cryopreservation protocols. In alginate encapsulated tissues thermal stability corresponds to a critical moisture content (MC) that must be achieved to prevent the formation of ice during cooling and rewarming, the latter being particularly important as glass relaxation and devitrification can result in the formation of lethal ice (12). Glasses created using alginate encapsulation/dehydration remain stable on passive rewarming at ambient temperatures so long as a critical lower MC is obtained during desiccation, in contrast vitrification solutions require optimal rewarming to avoid devitrification, glass fracturing and ice nucleation (12). As glass stability is dependent upon a crucial water content it is important to calibrate osmotic dehydration and desiccation treatments with survival. Using DSC, Benson et al. (8) found that encapsulated/air desiccated *Ribes* shoot meristems had stable Tgs on cooling and rewarming (at around -68°C and -60°C respectively) for a MC of ~25% (fresh weight basis). These thermal analyses were used by Block (16) and Benson et al. (12) to verify critical factors in alginate bead preparation; thus, stable glass formation on cooling and rewarming required a bead water content of 0.4 g water g^{-1} dry weight which corresponded with ~26% MC on the basis of fresh weight. In high humidity environments silica gel treatments are preferred to assure consistent desiccation. Under these conditions Sherlock et al. (143) revealed Tg profiles for encapsulated/dehydrated *Ribes* of -78°C to -51°C (cooling) and -88°C to -4°C (warming) at ~0.3 to 0.4 g water g^{-1} dry weight. Using DSC, Dumet et al. (42) studied the stability of different components of encapsulated-dehydrated *Ribes* shoot-tips and revealed minor thermal events on warming that were indicative of glass destabilization. However, this did not occur in the alginate or the *Ribes* shoot meristems when they were cooled and rewarmed separately. It might be expected that alginate encapsulated cells vitrified at very low water contents will have fewer, less mobile water molecules that are available to participate in nucleation, devitrification and ice growth on rewarming (9, 12).

Understanding the natural mechanisms that control desiccation tolerance in plant tissues that are sensitive to freezing can provide valuable insights into how to develop alginate-based cryostorage protocols (12, 141). Thermal analysis is a particularly valuable tool for the study of cryopreservation in tropical plant species that are recalcitrant to cryopreservation and for which the success of encapsulation/ dehydration or encapsulation/vitrification is critically dependent upon achieving MC and cellular viscosity at which stable glasses can be formed (12). For example, DSC profiles have been used to optimize alginate-based treatments for the cryopreservation of somatic embryos derived from the tropical tree *Azadirachta indica* (12). Similarly, Nadarajan et al. (103) constructed thermal profiles for encapsulated/vitrified shoot-tips of the recalcitrant seed producing tropical leguminous tree *Parkia speciosa,* demonstrating that trehalose moderated the thermal behaviour of the shoot-tips to favour glass stabilization and survival. The combination of alginate encapsulation, PVS2 and trehalose had the synergistic effect of reducing the molecular mobility of water, which may be advantageous in recalcitrant, desiccation sensitive tropical plant germplasm cryopreservation (103).

Once treatments are optimized the glasses formed in alginate encapsulated plant cells and tissues may be considered to be very stable and sucrose dehydration combined with desiccation limits the molecular mobility of water in the beads. Gonzalez-Arnao and Engelmann (51) propose that plant cells and tissues cryopreserved using encapsulation/dehydration can be rewarmed at ambient temperatures without risks of de-vitrification as the samples are sufficiently dehydrated to stabilize the glassy state. Thermal analysis studies do imply that this is the case, as long as treatments achieve a critically low, non-lethal MC before cooling and rewarming (8, 12).

Moisture profiles of encapsulated/dehydrated, cryopreserved microalgae (34, 63, 168) reveal that they are outside (ca. > 30% MC on a fresh weight basis) or close to the critical MC range usually required to form and stabilize the Tgs of encapsulated/dehydrated plant tissues (12). On cooling and rewarming this may risk devitrification in algal cells with the possibility of ice being formed at higher moisture levels. This may explain why the cryopreservation of algae using encapsulation/dehydration requires an additional controlled rate cooling step (34, 168) presumably because cryo-dehydration of the remaining water is necessary to achieve a stable vitrified state. Harding et al. (55) applied DSC to study vitrification in encapsulated/dehydrated *Euglena gracilis*, revealing a Tg at -75°C on cooling in cells that had been dehydrated in sucrose and air desiccated for 3 h to a residual MC of 25% (fresh weight). In contrast, Hirata et al. (63) demonstrated the survival of several species of encapsulated algae and cyanobacteria without the need for controlled rate freezing to be applied after the encapsulation/dehydration.

Clearly alginate encapsulation as applied to phytodiversity cryopreservation has promising future applications, although progress to date has been advanced mainly through empirical research. Future developments will require a greater fundamental knowledge of cryobiology and the stress physiology that underpins alginate-based cryopreservation and an understanding of natural adaptations to cold stress as well as those incurred during cryostorage (9, 12, 55, 141).

5. ALGINATE ENCAPSULATION FOR FUNGAL CRYOSTORAGE

Methods of encapsulation have been used to entrap microorganisms for many years. 'Immobilised' fungi have been used by the biotechnology industry to produce novel products and commercial enzymes (78), for formulation of biological control agents (32, 110), for carriers of mycoherbicides (169) and as sources of inocula for ecomycorrhizal fungi (95). However, unlike other cell types, alginate encapsulation has not been widely adopted for the cryopreservation of fungi. This is primarily because the majority of filamentous fungi can be preserved using standard approaches to cryopreservation that involve the application of a controlled cooling rate in association with an appropriate cryoprotectant, typically 1°C min^{-1} with 10% glycerol solution (126). Further, for most culture collections, encapsulation is significantly more laborious than traditional cryopreservation approaches and the need for sterility is imperative as the perceived increased risk of sample contamination due to the many manipulative steps that are involved in the encapsulation process. For cryopreservation recalcitrant fungi and the heterotrophic filamentous chromists, alternative approaches are required, and there have been several reports of encapsulation being utilised for this purpose. A basic encapsulation approach was used for the preservation of the dry rot fungus *Serpula lacrymans* (125). This economically important basidiomycete fungus is notoriously difficult to maintain. Like other basidiomycetes it is only the 'mycelial' state that can be preserved and this state is susceptible to ice damage during cryopreservation. Although, the alginate beads were not subject to dehydration, the author found that encapsulation in calcium alginate significantly improved post-storage viability. A more advanced encapsulation/dehydration approach was used by Wood et al. (172) for the simultaneous preservation of orchid seed (*Dactylorhiza fuchsia* and *Anacamptis morio*) and its fungal symbiont (*Ceratobasidium cornigerum*). After encapsulation, the biological material was subjected to pretreatment with sucrose and subsequent drying before cryopreservation. It was found that embryo growth of both orchids and fungal development were not detrimentally affected when the beads were pre-dried to ca. 20% MC with viability of the seed and the fungus remained unchanged during 30 days storage at -196°C. The results

indicated opportunities for utilising both simultaneous cryopreservation and encapsulation as a conservation tool for diverse taxa and has since been used for other terrestrial orchid relationships (147). Cryopresevation of arbuscular mycorrhizal fungi is also problematical. Unlike filamentous fungi, many mycorrhizal fungi cannot be cultured *in vitro*, so researchers have turned to alginate encapsulation approaches (79, 80) as this has the added advantage in that it can be used as a direct source of inoculum (151).

Despite success with mycorhizza, there are very few published reports about the use of encapsulation for cryopreservation of filamentous fungi. However, a more in depth study was undertaken, where an encapsulation vitrification protocol was trialled across five European Culture Collections (3). The protocol was based on a regime originally developed for microalgae through the EU Cobra project (57) and was modified for use with fungi. A suite of representative fungi from the taxonomic groups Zygomycota, Basidiomycota, Ascomycota and some heterotrophic chromists were selected and then encapsulated using a technique which involved encapsulation of mycelium into calcium alginate, stepwise dehydration using sucrose gradient solutions and air drying, before plunge cooling in LN. The study also compared the genetic stability of samples subjected to encapsulation with those subjected to standard cryopreservation approaches. It was found that for most fungi, encapsulation-vitrification cryopreservation can produce viable, morphologically 'typical' cultures, therefore negating the need for control rate cooling. However, some evidence of genetic instability was detected and this warranted further investigation. Of particular note was the successful encapsulation and recovery of the heterotrophic chromists *Phytophthora citrophthora* and *Saprolegnia diclina* (71, 144). Heterotrophic chromists are members of the Stramenopila and are particularly vulnerable to ice damage as the mycelial filaments are aseptate. When subjected to traditional cryopreservation regimes, ice seeds down the hypha, thus the removal of water during the encapsulation-vitrification procedure negates the prospects of lethal ice formation.

While osmotic dehydration is the standard approach for fungal encapsulation cryopreservation, some workers advocate omitting a stepwise dehydration approach and simply suspending beads in cryoprotectants such as glucose and lactose. This was applied to strains of *Malassezia* spp. and the zygomycetes species *Rhizopus* and *Mucor*. It was concluded that the use of 23% glucose or lactose at -80°C in a sodium alginate cell immobilisation system is efficient for cryopreserving zygomycetes, although *Malassezia* spp. did not survive beyond (124).

There is still much potential for the application of encapsulation dehydration techniques to cryopreservation recalcitrant fungi and heterotophic chromists and related organisms, particularly those susceptible to ice damage. The method may also be useful for preservation strategies for the microbiome and has an application in laboratories that do not have access to controlled rate cooling equipment.

6. ALGINATE ENCAPSULATION CRYOPRESERVATION APPLIED TO ANIMAL AND HUMAN CELLS

The development of AECryo in animal and human cells in some ways mirrored the progress made in plant cell technologies, but arrived some 10 years later. Alginate encapsulation was indeed under study in animal cell fields by the late 1980's, but from a different perspective – the use of the biocompatible hydrogel to prevent immune rejection of transplanted animal cells such as Islets of Langerhans or hepatocytes in cell transplant models (22, 97, 107). In these situations, the alginate hydrogels were found to allow diffusion of small molecules and metabolites into and out from the transplanted cells, which could be taken as an early pointer that for cryopreservation, the necessary movement of cryoprotectants and water should also be achievable. Pursuing these ideas, Woods and colleagues (173) undertook experiments to compare cryoprotective additive (CPA) permeation into human Islets of Langerhans either indirectly or after prior alginate encapsulation. Results indicated that there were small but important differences in CPA permeation parameters, which should be taken into account when applying AECryo.

In the cell transplantation field, several applications combined alginate with secondary agents such as poly-L-lysine and chitosan for improving stability of the supporting hydrogel matrix (54, 107, 112). Islets remained a focus of attention and it was shown that rat Islets could be cryopreserved using DMSO, AECryo with

alginate – poly-L-lysine and slow cooling approaches, with a subsequently good function demonstrated as *in vitro* release of insulin to a glucose challenge (26). Alongside the interest in animal cell biotechnology, AECryo was applied for cryopreservation of stably transfected human kidney cells, again using DMSO and slow cooling (150). Flow cytometry revealed no AECryo induced changes in cell ploidy with a good recovery of therapeutic protein synthesis following thawing. Application of AECryo to both primary rodent hepatocytes or to an immortalised human liver cell line using standard slow cooling techniques was equally successful (86), with improved survival in a rat model of fulminant liver failure after transplantation of thawed encapsulated liver cells. Similarly, long-term storage of AECryo rat hepatocytes was shown to be compatible with maintenance of drug transporting properties (73). For the majority of animal cell studies, alginate encapsulation required to produce beads with diameters within the range 200–400 µm, with alginate concentrations between 1–2% w/v. For example, for AECryo of porcine primary hepatocytes, 400 µm alginate – poly-L-lysine beads were used to reverse fulminant hepatic failure in a mouse model system (98). Again, application of slow cooling with CPA DMSO was found to be successful when transplanting the thawed, bead-encapsulated hepatocytes. Further studies on AECryo of rat and porcine Islets of Langerhans compared alginate – poly-L-lysine and alginate – protamine heparin beads, with slow cooling and DMSO system. Cryobanking was carried out for > 400 days in some cases, whilst post-thaw recoveries were higher when alginate encapsulated Islets were compared with non-encapsulated cells (5). Reports of successful cryopreservation of Islets applying AECryo and standard slow cooling protocols continue to be published (135), with certain refinements such as AECryo of single Islet containing beads (28), which can improve the understanding of robust numerical cell recovery data post cryopreservation, which can be difficult when multiple cell numbers have been encapsulated at the start of the process.

Reproductive cryobanking of animal or human cells is one of the largest end users of cryostorage. Within that discipline, interest in AECryo has also recently developed. Song and colleagues (148) discussed the possible benefits of encapsulation strategies in their review on future developments in cryopreservation in reproductive medicine. The possibility of applying AECryo techniques for cryopreservation of sex sorted semen has recently been discussed (149). The concept of alginate encapsulation to support the *in vitro* growth of isolated follicles from human ovarian tissue has also recently been discussed (178), but cryopreservation has not yet been reported. Similarly, alginate encapsulation with growth factor-laden nanoparticles has been suggested to improve graft success of mouse immature testicular tissues (134, 135) after initial tissue cryopreservation, but again AECryo as a discrete step has not yet been reported. It is likely that AECryo will become more widely applied in reproductive cryopreservation in the future.

Alginate encapsulation for mammalian stem cells

Since 1964, when Thomas M. S. Chang developed a method for microencapsulation of biological material in natural polymers (25), cell microencapsulation in alginate has been widely investigated mainly in the field of transplantation, because the semi-permeable alginate matrix protects encapsulated cells from the host immune system. It also came to be understood that alginate encapsulation could offer advantages for manipulating cells undergoing cryopreservation. The approach seems to be feasible for allogenic and xenogenic transplantation of encapsulated cells. However, the number of primary, usually terminally differentiated cells, sufficient for therapeutic efficacy, is limited by the complexity of the isolation procedures and donors' organ availability. Therefore, an attractive alternative is stem cells due to their ability for self-renewal and multilineage differentiation. Stem cells are now found in nearly all tissues throughout the body, their properties are well characterized and by differentiation capacity they may be classified as pluripotent or multipotent.

<u>Pluripotent stem cells</u>

Pluripotent stem cells include embryonic stem cells (ESCs), derived from the inner cell mass of the pre-implantation blastocyst (46, 161), germ stem cells (GSC) derived from primordial germ cells that arise in the late embryonic and early fetal period of development (142) and induced pluripotent stem cells (iPSCs), obtained by reprogramming somatic cells to a pluripotent state (153). All these pluripotent stem cells can expand indefinitely *in*

vitro and differentiate into all three germ lineages, consequently giving rise to cells from all tissue types. ESCs, GSCs and iPSCs demonstrate similar phenotype, growth and differentiation patterns. So, results obtained on one type of cells can be easily translated to all.

Numerous studies have demonstrated that two-dimensional (2D) culture systems routinely used for expansion of these stem cells do not adequately simulate the *in vivo* microenvironments of stem cell niches (82) and limit the control of cell growth and differentiation from external sources. Moreover, pluripotent ESCs expanded in 2D culture systems show low cell recovery yields and high rates of uncontrolled differentiation were obtained after conventional slow-cooling cryopreservation protocols (60). In comparison with the low survival rates obtained with slow-cooling cryopreservation, vitrification protocols provide higher cell viability, particularly regarding murine ESCs (83, 180). These limitations have resulted in low production yields and have stimulated the development of efficient, scalable and cost-effective stem cell expansion processes (113).

Development of 3D systems for expansion of human ESCs as aggregates or after immobilizing them on microcarriers, in combination with usage of bioreactors, has provided the possibility to obtain homogenous cultures and enhance cell recovery yields (70, 139). Alginate microcapsules can be considered as a 3D specific microenvironment for pluripotent stem cells supporting cell proliferation and control of differentiation from the external environment. ESCs encapsulated in 1.1% alginate hydrogels retained their pluripotency without passaging or embryoid body formation during culture in basic medium for a period of up to 260 days and could differentiate when they were subsequently cultured in a conditioned environment (145). Moreover, encapsulation in alginate enhanced the differentiation of ESCs into hepatocytes (83), insulin-producing cells (170), cardiac cells (69), retinal and neuronal lineages (37, 66). In addition, alginate encapsulated pluripotent stem cells are more resistant to mechanical forces taking place in stirred tank bioreactors.

Cryopreservation of ESCs as single cells, aggregates or immobilized on microcarriers has been the subject of many reports, and their results well analyzed in reviews (65, 99). In some respects, cryopreservation of alginate encapsulated pluripotent stem cells is still poorly investigated, while cryopreservation can be easy integrated into the total biotechnological process, included expansion, cryopreservation and rehabilitation of cells after rewarming. A mathematical model which couples the mass transport of DMSO into a cell-seeded spherical construct and cell membrane transport into ESCs was applied to predict overall cell survival rate after conventional cryopreservation (130). Predicted results were confirmed by the experimental data which show survival of about 60% after rewarming of murine ESCs cryopreserved using -1°C per min slow-rate cooling with 10% DMSO as CPA within 1.2 mm alginate capsules. Cryopreservation under the same conditions using murine ESCs within adhesive alginate microcapsules, where alginate was modified by coupling arginine-glycine-aspartic acid-serine (RGDS) providing cell attachment to the substratum, led to even more encouraging results (129). Cells cryopreserved in RGDS-alginates had survival of 93±2% (trypan blue exclusion assay), and significantly higher metabolic rates than both cells cryopreserved in suspension and those encapsulated in unmodified alginate. After rewarming murine ESCs cryopreserved in RGDS-modified alginate microspheres maintained the expression of stem cell markers and pluripotency assessed by differentiating them along the three lineages.

However, conventional cryopreservation of human ESCs demonstrated less optimistic results. In one study (90) a comparative evaluation of different procedures of slow-rate cooling for cryopreservation of intact, adherent human ESCs as colonies or clumps entrapped in alginate has been carried out. Conventional cryopreservation with 10% DMSO included slow-rate cooling in three different media, which resulted in extensive loss of membrane integrity and metabolic activity within 24 h post-rewarming. No additional cryoprotective effect was observed when the apoptosis inhibitors Z-VAD-FMK or/and ROCK inhibitor Y-27632 were added either to the cryoprotective medium or after rewarming. Alginate encapsulation did not provide further protection to low-rate cooled ESCs as either colonies or clumps. Although reduced cell detachment from the culture surface for alginate encapsulated ESCs colonies was observed immediately after thawing, within 24 h all cells presented damaged membranes regardless of the alginate entrapment. In another

study by Serra et al. (140) ESCs were microencapsulated in alginate microspheres with a diameter of approximately 500–700 μm as: i) single cells; ii) aggregates; and iii) immobilized on Cytodex microcarriers coated with Matrigel as previously has been reported by Nie et al. (104). After conventional cryopreservation in a medium containing 10% DMSO using a low rate (-1˚C/min) cooling protocol, storage in the gas phase of a LN, rapid warming and stepwise washing out DMSO, ESCs were cultured for the next 9 days. Results showed that in aggregate culture, alginate microencapsulation did not prevent cell death immediately after rewarming. On the contrary, microencapsulated ESCs immobilized on microcarriers presented higher cell viabilities and cell recoveries after cryopreservation. These results indicate that for the cryopreservation of alginate encapsulated human pluripotent stem cells, the conventional slow cooling protocols should be further optimized.

Multipotent stem and progenitor cells

Multipotent stem cells have more limited self-renewal and differentiation capacity than pluripotent stem cells; usually multipotency is restricted to producing the differentiated cell types of the tissue in which they reside. Common multipotent stem cells include neural stem cells (NSCs), mesenchymal stem/stromal cells (MSCs) and hematopoietic stem cells (HSCs). NSCs have specific phenotypes and are able to differentiate into neurons, astrocytes, oligodendrocytes and microglia (131, 138). In culture NSCs proliferate forming non-adherent clusters named neurospheres. As a source of NSCs for research and cell based therapy, neurospheres have been a subject of numerous multidisciplinary studies, including cryobiology. Nevertheless, there is only one report aimed at the development of a strategy for neurosphere cryopreservation using alginate encapsulation (89). Neurospheres prepared from fetal rat brain were encapsulated at the 5th day of culture and cryopreserved at day 19. Alginate encapsulation prevented fusion of small neurospheres and did not affect the viability, proliferation and glycolytic metabolism of the cells or the development of neurospheres during pre-cryopreservation culture. Alginate encapsulated and non-encapsulated neurospheres were equilibrated in a cryopreservation solution containing 10% DMSO for 30 min at 4°C. Samples were cooled at -1°C/min to -80°C, then stored in the vapor phase of LN for 1–2 weeks. It was shown that cryopreservation of non-encapsulated neurospheres resulted in loss of spherical shape and cell disintegration. For encapsulated neurospheres, no significant fragmentation or loss of spherical shape was observed after cryopreservation. Cell survival of both encapsulated and non-encapsulated neurospheres was high immediately post-warming and decreased after the first 24 h of culture. Subsequently alginate encapsulated neurospheres were able to recover during post-rewarming culture and maintain capacity for neuronal and glial differentiation.

Mesenchymal stem/stromal cells (MSCs) can differentiate into cells of bone, cartilage, muscle, fat and other tissues, which makes them attractive for regenerative medicine and tissue engineering. MSCs are adherent cells and in monolayer culture they grow as flat colonies of fibroblast-like cells. Alginate has weak adhesive properties and after encapsulation in alginate hydrogel MSCs become round-shaped and reversibly stop proliferating (115).

Successful cryopreservation of alginate encapsulated MSCs provides cryobanking of the cells and allows to avoid cell aging, and prevent loss of their ability to perform multilineage differentiation. In a recent report (115) human bone marrow MSCs, either encapsulated in alginate microbeads with sizes over the range of 500-1000 μm or as cells in suspension, were cryopreserved with 5% and 10% DMSO using conventional 2-step slow cooling (protocol 1). The viability and metabolism of MSCs in alginate microbeads following cryopreservation with 5% DMSO were lower than those cryopreserved with 10% DMSO. MSCs in suspension were more resistant to cryopreservation than alginate encapsulated cells when cryopreserved with 5% DMSO, although when using 10% DMSO, no differences were detected. Comparison of the viability and metabolic activity of MSCs cryopreserved either in alginate or as cell suspensions with 10% DMSO using protocol 1 (2-step cooling), protocol 2 (3-step slow cooling with induced ice nucleation) or protocol 3 (rapid 1-step cooling), showed that the highest viability and metabolic rates were obtained following cryopreservation of MSCs in alginate microbeads by protocol 2 (with controlled ice nucleation) (115). After cryopreservation by protocol 2, alginate encapsulated MSCs were capable for multilineage differentiation towards osteogenic,

adipogenic and chondrogenic lineages. Positive effects of initiation of ice crystallization can be explained by removal of critical supercooling events, which may negatively impact on the MSCs in alginate microbeads during slow cooling. This strategy has been successfully used for cryopreservation of alginate encapsulated chondrocytes (1), and transformed human embryonic kidney cells (150) and liver cells (93). Results similar to the ice seeding effect were obtained after reduction of the cooling rate to 0.5°C/min. In this case survival of human dermal MSCs after cryopreservation with 7.5% DMSO within alginate microbeads was 81 ± 4% and did not differ from that in suspension (157). The difference in DMSO concentrations (10% and 7,5%) used in the studies (115, 157) was obviously not important, because in another report (52) it has been shown that MSCs derived from common marmoset monkey and encapsulated in alginate beads could be cryopreserved with equal efficiency using slow-cooling protocol with 7.5% and 10% DMSO as a cryoprotectant.

Results on human and primate MSCs do not confirm the positive opinion of the cryoprotective properties of alginate hydrogel obtained for example during rapid cooling of encapsulated hepatocytes (6, 77). Cryopreservation of human MSCs with rapid cooling even with 10% DMSO resulted in critical damage to the encapsulated MSCs (115). Moreover, after rapid cooling, changes in the structure of the alginate gel have been observed, which were reflected in a loss of transparency and shape of the alginate microbeads; these effects may have resulted from growth of ice crystals within the polymer gel matrix during rapid cooling. Similar responses of alginate microbeads' integrity and encapsulated therein CV1 line cells during either rapid or slow cooling were described by Heng et al. (61).

The integrity of alginate beads during rapid cooling is a requirement for the successful cryopreservation of alginate encapsulated cells via vitrification where outcomes, besides general conditions such as cooling rate and sample volume, will depend on microbeads' size and composition. For example, rapid-cooling cryopreservation protocols included plunging the cryovials with cells encapsulated in 300-400 μm alginate microbeads with high DMSO concentrations (3.5-4.5 M) directly into LN and did not enable completely the prevention of microbead disruption and also resulted in low post-warming cell viability (<10%) (61). In addition, inclusion of 0.25 M sucrose or 20% Ficoll did not significantly improve neither post-warming cell viability nor microcapsule integrity. On the other hand, vitrification in small (~100 μm) alginate microbeads using 400 μm quartz microcapillaries resulted in their post-warming intactness in the presence of 1.5 M or higher DMSO concentrations (181). Mouse MSCs encapsulated in small alginate microbeads and derived from mouse embryos had 2-times higher post-warming survival than non-encapsulated cells (89±3% and 42±4%, respectively). In the later report by Huang et al. (64) vitrification of murine ESCs and human adipose-derived MSCs encapsulated into small alginate microbeads (~220 μm) using small sample volume (<2.5 μl) and correspondently high cooling/warming rates in culture medium with 1.3 M trehalose and 2 M 1,2-propanediol as the non-penetrating and penetrating CPA, respectively, compromised neither microbead integrity nor the specific functional properties of the cells assessed by expression stem cell markers and their differentiation ability (64). Moreover, the authors revealed that alginate hydrogel microencapsulation could effectively inhibit devitrification during warming, thus reducing concentrations of required penetrative cryoprotective agents. However, the vitrification protocol in small microbeads and in small sample volumes has not yet been performed for the practical application to bank stem cells where large volumes are often preferred.

Vitrification of human MSCs in large alginate microbeads with sizes over the range of 1100-1200 μm in 0.5 ml of multicomponent vitrification solution (VS) comprised of 10% DMSO, 20% ethylene glycol, 20% 1,2-propanediol and 0.5 M sucrose using standard cryovials was studied (164). High post-warming viabilities, metabolic activity and two-lineage differentiation processes of MSCs comparable with non-encapsulated cells (and without loss of microbead integrity) was achieved after the significant extension of exposure time to the cryoprotective agents. Successful vitrification of porcine MSCs in large (800-1000 μm) alginate–fibrin microbeads has been achieved in a minimal volume of VS composed of 40% ethylene glycol and 0.6 M sucrose (15). The wrinkled surface of the alginate-fibrin microbeads observed after warming using scanning electron microscopy was non-detrimental to cell viability and functionality, as

confirmed by live/dead viability staining and metabolic assay. Recently the same protocol and VS was applied for the vitrification of human bone marrow MSCs attached on the surface of alginate microbeads coated by chitosan and collagen type I (175). It was shown (175) that the ability of MSCs cultured on the surface of microcarriers to proliferate was not affected by vitrification and it was significantly improved after vitrification compared to conventional slow cooling during continuous culture. Despite the benefits of vitrification the adaptation of cell cryo-banking towards clinical grade Good Manufacturing Practice (GMP) standards is required.

(7): ALGINATE ENCAPSULATION FOR STORAGE OF CELLS AT NON-FREEZING AND POSITIVE TEMPERATURES

For some applications and for cryopreservation of recalcitrant species, tissues and cells, biopreservation in the non-frozen state has certain advantages. In the case of hydrated, human clinical biomaterials this concerns storage in the liquid (ice-free) condition. In contrast the successful preservation at positive temperatures of encapsulated, viable bioresources representing the wider spectrum of biodiversity usually requires a reduction in water content. This is optimized using osmotic treatments together with chemical and evaporative dehydration and desiccation (see Figs. 2 and 3) these regimes can simulate an organism's natural response to seasonal cues in nature, especially cold acclimation and seasonal dormancy which are adaptive survival strategies (9, 49). Generally, biopreservation at positive temperatures is aimed at allowing the short-term storage and distribution of biomaterials to end-user laboratories and it may be attractive to avoid the extra costs and complexities associated with cryogenic preservation. This can be especially relevant in countries or regions where reliable access to cryogens such as LN, or electrical power to maintain low temperature freezers may be problematic. Similarly, the logistics of transferring and stabilizing at sub-zero temperatures viable samples from remote field sites can be problematic (9), in these situations, alginate encapsulation may have added benefits to already developed storage and handling regimens. Such approaches have been applied for a range of taxa although in historical terms, non-freezing AECryo technologies were developed first for plant germplasm deriving from the focus of interest in artificial seeds.

Alginate encapsulation for the storage of plant propagules at positive temperatures

Alginate artificial seed technology as applied to somatic embryos (91) can be adapted for other plant propagules including meristems (48, 68) and nodal segments (31). This permits the medium-term storage of a range of plant germplasm which can be achieved when bead MC and treatments are optimized to prevent deterioration or premature development of the propagules during storage or transport at positive temperatures. Assuring the stability and longevity of alginate encapsulated propagules at non-freezing temperatures requires the careful optimization and validation of medium-term storage parameters with acceptable recovery outcomes (24, 68, 102, 108). The conservation of encapsulated cotyledonary nodal segments from *Cedrela fissilis* for 3 months at 25ºC was possible with 90-100% recovery, although the propagules deteriorated by 6 months storage and their viability was reduced to 6-8% after 9 months (31). *Cedrela fissilis* is an endangered tree native to the Brazilian South Atlantic Forest and this study demonstrates the potential use of encapsulation at positive temperatures for the *ex situ* conservation of at risk species. In the case of economically significant plants, Ikhlaq et al. (68) applied alginate encapsulation in artificial endosperm solution to shoot meristems of *Olea europaea*; acceptable levels of recovery were achieved after 45 days storage at 4ºC. Alternatively, Nower et al. (106) manipulated the storage longevity of alginate encapsulated meristems of *Pyrus communis* using growth retardants, an approach that may be useful for the synthetic seeds of species sensitive to low temperatures. Plant regeneration following medium term storage of alginate encapsulated somatic embryos, embryoids and embryogenic masses at 4ºC for 30 - 60 days has been achieved for *Citrus reticulata* (4) and *Pistachio vera* (108) respectively. Castillo et al. (24) demonstrated that alginate encapsulated embryos of *Carica papaya* survived 85 days storage under low light at 10ºC. In all cases alginate encapsulation improved post-storage survival of embryos compared to non-encapsulated controls.

Whilst the storage of plant germplasm at positive temperatures has many advantages assuring the stability of plants regenerated from synthetic seeds is important, particularly as *in*

vitro culture and preservation at non-cryogenic temperatures can predispose plants to instability and genetic variation (56). Faisal et al. (48) assessed the genetic fidelity of plants regenerated from encapsulated micro-shoots of *Rauvolifia serpentina*, which were stored for 4 weeks at 4ºC. Random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) marker studies confirmed plantlets regenerated from the synthetic seeds of this medicinal plant were comparable to those of mother plants.

Alginate encapsulation for hypothermic or positive temperature preservation of animal cells

Efficient storage and transportation of the cells without affecting their functional activity and specific properties is an essential factor in any clinical and laboratory practice. For short-term storage, it is often not necessary to use technically complex and costly cryobiological methods demanding of specialized equipment, trained staff and the use of toxic cryoprotectants, which should be washed out before use. At the same time, the period of time when cells and tissues can be stored at low positive temperature (4-23°C) is limited and this encourages the search for new and optimization existing approaches to extend the time without affecting the quality of the biological material.

One of these approaches is the encapsulation of cells in a semipermeable alginate hydrogel. It has been shown that cell encapsulation can improve their viability during storage at low positive temperature without the use of specialized media and additives. Thus, in the study from Chen et al. (27), human MSCs and murine ESCs were successfully stored inside alginate hydrogels for 5 days under ambient conditions in an air-tight environment. Viability of the cells extracted from alginate gel compared favourably to cryopreservation: 74% for ESC and 80% for human MSC. More importantly, the subsequent proliferation rates and detection of common stem cell markers from human MSCs and ESCs retrieved from alginate hydrogels were also comparable to results gained following cryopreservation. Tarusin et al. (156) carried out a comparative study of viability, function and capacity for multilineage differentiation of human dermal MSCs, stored at various temperatures either as cell suspensions or within alginate microbeads. It has been shown that the storage of the MSCs at temperatures of 4, 22 and 37°C in sealed cryovials in suspension for 3 days resulted in a decrease of the viability, attachment properties and metabolic activity. MSCs after storage within alginate microbeads at 22 and 37°C showed a high viability (78 and 87%, respectively), retained the attachment properties (62 and 70%), metabolic activity (79 and 75%) and ability to differentiate towards osteogenic and adipogenic lineage.

In another report, Swioklo et al. (152) examined whether hypothermic (4°C–23°C) preservation of human adipose-derived MSCs could be improved through their encapsulation in 1.2% calcium alginate. Alginate encapsulation improved the recovery of viable cells after 72 h of storage. Viable cell recovery was highly temperature dependent, with an optimum temperature of 15°C. At this temperature, alginate encapsulation preserved the ability for recovered cells to attach to tissue culture plastic on rewarming to normothermia, further increasing its effect on total cell recovery. On attachment, the cells were phenotypically normal, displayed normal growth kinetics and maintained their capacity for trilineage differentiation. The number of cells encapsulated did not affect viable cell recovery nor did storage of encapsulated cells in a xeno-free, serum-free, current GMP-grade medium.

The influence of alginate encapsulation on the preservation of the cells in the storage conditions at 4°C without the use of specialized media and supplements remains controversial. Swioklo et al. (152) observed that at 4°C during 72 h, non-encapsulated MSCs demonstrated a dramatic decrease in viability; compared to a yield of only about 18% of viable cells initially stored, encapsulated cells exhibited a 3.7-fold increase in the number of viable cells recovered compared to control. Experiments on the effects of hypothermic storage (4°C) up to 24 h upon cell viability and enzyme release in 1 or 1.5 % alginate beads containing baby hamster kidney clones overexpressing α -L-iduronidase (IDUA) were performed (96). It was shown that the 1% alginate group presented a small but not significant reduction in enzyme activity, while the 1.5% alginate group presented values for IDUA activity threefold those of the pre-storage levels.

Mahler et al. (84) analysed the protective effects of alginate encapsulation on rat hepatocyte viability, cell yield, and both mitochondrial and other cytoplasmic functional activities, and apoptosis after either 24 or 48 h of

hypothermic storage. Decrease in viability was recorded at 4% and 13% (24 h at 4°C), 15% and 33% (48 h at 4°C) for encapsulated and free suspended hepatocytes, respectively. The mitochondrial enzymes, EROD and GST activities were better preserved in encapsulated than in free suspended hepatocytes (84). Hypothermic storage processes were found to induce early caspase-3-like activities, being always much lower in alginate encapsulated hepatocytes. Thus, cold-induced apoptosis in hepatocytes can be significantly reduced following their encapsulation within alginate gel beads and this is associated with an improvement of both their viability and function. Analysis on the effects of alginate concentration, storage conditions and period of encapsulation on cell viability to examine the suitability of a calcium alginate hydrogel for preservation of corneal epithelial cells was carried out by Wright et al. (174). It was demonstrated that 0.6% (w/v) alginate gel composition allowed storage of corneal epithelial cells most effectively in either a hypothermic (4°C, atmospheric CO_2 and humidity levels) or cell culture environment for up to 5 days. Modification of the macrostructure of a calcium alginate gel from an amorphous mass to a thin disc enhanced corneal epithelial cell viability, limited cell proliferation and overcame the reduction in cell viability observed with increases in alginate concentration. The authors noted that viability of cells encapsulated within calcium alginate gels was influenced by both pore size and mechanical properties (174). The extent to which these structural features affect cell viability may be manipulated by modifying proportions of alginate and porogen (HEC) in gels. All these data support a beneficial effect of alginate upon cell viability and function after hypothermic storage (4-8°C).

In contrast, other studies failed to show a positive effect of alginate encapsulation at hypothermic storage (4°C) or in attempts to extend the storage periods without using specialised additives (see following discussion). Hypothermic storage presented as harsh conditions for encapsulated (in alginate-PLO-alginate microcapsules) buffalo rat liver cell line propagation and the secretion of metabolic products (179). When the storage temperature was 4°C, there was no obvious difference between cells re-cultured for 1 day and 3 days, cell viability was very low and the remaining living cells were hardly able to propagate. Hypothermic storage of human dermal MSCs within alginate microbeads at 4°C resulted in a gradual decrease of viability during 3 and 7 days; and at the end of storage no significant differences between cells in suspension or in alginate microspheres were observed (156, 159). Despite the fact that investigators in some studies managed to achieve a positive result of encapsulation at hypothermic storage temperatures (4-8°C), one must conclude that these results were obtained only after short time windows. Perhaps more prolonged storage of cells without the use of specialized media and additives will have a dramatic effect on the cells' fate irrespective of alginate encapsulation.

For this purpose, in the report by Tarusin et al. (158) the effect of hypothermic storage at 4°C of human MSCs as suspension or encapsulated in alginate microspheres was studied using sucrose-based solution (SBS) (74) and the University of Wisconsin solution (UW). It was shown that after 7 days at hypothermic storage in SBS and UW, suspensions of MSCs and MSCs encapsulated in alginate microspheres retained high levels of viability (60-80%), adhesive properties, metabolic activity and the ability to induced adipogenic and osteogenic differentiation. Thus, this confirmed that the use of preservation solutions SBS and UW can prolong hypothermic storage of the MSCs. However, the positive influence of alginate encapsulation compared to cell suspension was not identified.

Other factors may impact on the viability of the alginate encapsulated cells at positive temperatures during storage. Thus, the purpose of the study by Dontchos et al. (40) was to determine whether an increase in pH decreased chondrocyte viability during cold storage in alginate microbeads and whether equilibration of Dulbecco's modified Eagle's medium (DMEM) in 5% CO_2 normalized pH and increased chondrocyte survival during storage at 4°C. After 5 days of storage in alginate microbeads at +4°C, chondrocyte necrosis was higher when stored in ambient air than if equilibrated with 5% CO_2. These data show that an increase in pH decreased bovine chondrocyte viability when refrigerated at +4°C in DMEM, and that optimization of CO_2 with normalized pH did improve chondrocyte viability during cold storage in DMEM.

Any positive effect of alginate encapsulation on cell state during storage may be based on a number of up to now unidentified

factors. Protective effects may be caused directly by the alginate matrix, but can result from an indirect influence. Hypothetically, by stabilizing plasma membranes, the alginate gel could provoke integrin ligation, which disrupts the integrin-caspase complex and thus increases survival, thereby revealing an unexpected role for entrapment in alginate beads in the regulation of apoptosis (84). Further study is needed to clarify this hypothesis because not all cells are subject to integrin-mediated death, and not all integrins are pro-apoptotic. Indirect effects of the alginate hydrogel may result from its mass transfer and diffusive characteristics confirmed for different molecular weight solutes (116). Thus, the spatial separation of the cells from each other could positively impact by decreasing proteolytic enzymes released and diffusion of factors from the dead cells to viable ones. One cause of a positive effect of alginate encapsulation also may result from reversible total metabolic inhibition and decrease in mitochondrial membrane potential, as it has been shown in culture of encapsulated fibroblasts (67). In support of this mechanism it could be suggested as a weak, positive cell response to encapsulation during storage at 4°C, where independently of the presence of the alginate hydrogel, cells are already in the low metabolic state (159).

The overall results discussed here show that the selection of temperature conditions, environment and physicochemical properties of alginate will allow us to develop a technology which may significantly prolong the effective short-term storage of cells for their safe transport and exchange between clinical and experimental centres. Muller et al. (102) debate the possibility of ambient, positive temperature storage of nucleic acids in terms of logistics, safety, and cost and conclude that it is of value only if the stability of the samples is adequate. Although there are advantages associated with the storage and transport of alginate encapsulated biomaterials and viable bioresources at positive temperatures, it is recommended that these biopreservation methods are validated before they are used routinely. In particular, their evaluation should include the future proofing of post-storage quality and stability (102).

CONCLUSIONS

Alginate encapsulation cryopreservation was originally pioneered for the *ex situ* conservation of plant genetic resources and AECryo protocols have now been validated for routine use in some plant genebanks and biorepositories. The encapsulation of viable cells, tissues, organs and organisms in alginate provides an alternative, low temperature storage method enabling the successful cryopreservation of bioresources representing diverse taxonomic groups and, more recently, clinical and therapeutic biospecimens. AECryo has similarly been applied in algal, microbial, fungal and protist culture collections and is particularly useful for the storage of organisms that have proved difficult (recalcitrant) to cryopreserve using traditional protocols. Moving forward, there remain important factors to consider alongside further expansion of AECryo. Different alginate sources, with different polymer mixes (and therefore different physical characteristics), may provide subtle differences in successful stability in the cryopreserved state. In terms of AECryo safety, bio-contamination of alginate may be more or less important. For example, for human application, alginate may require stringent purification steps to remove bio-contaminants. Some of these factors have been recently reviewed (109). In AECryo for plant, algae and fungi, it seems so far, that contamination introduced via poor containment, inadequate aseptic techniques, cross-contamination and use of non-axenic cultures (e.g., that contain systemic endophytes) before cryopreservation have been the main identified problems, rather than the alginate itself. Thus, we can conclude that, almost three decades after it was first pioneered, AECryo technology currently enables the long-term storage of bioresources across diverse clinical and non-clinical sectors, including agriculture, biotechnology, conservation, forestry, horticulture and environmental sciences.

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