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PAPER

A cryopreservation method for bioengineered 3D cell culture models

Alba Herrero-Gómez, Marc Azagra and Irene Marco-Rius*

Institute for Bioengineering of Catalonia, Baldiri i Reixac 10-12, 08028 Barcelona, Spain
* Author to whom any correspondence should be addressed.

E-mail: imarco@ibecbarcelona.eu

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Abstract

Technologies to cryogenically preserve (a.k.a. cryopreserve) living tissue, cell lines and primary cells have matured greatly for both clinicians and researchers since their first demonstration in the 1950s and are widely used in storage and transport applications. Currently, however, there remains an absence of viable cryopreservation and thawing methods for bioengineered, three-dimensional (3D) cell models, including patients' samples. As a first step towards addressing this gap, we demonstrate a viable protocol for spheroid cryopreservation and survival based on a 3D carboxymethyl cellulose scaffold and precise conditions for freezing and thawing. The protocol is tested using hepatocytes, for which the scaffold provides both the 3D structure for cells to self-arrange into spheroids and to support cells during freezing for optimal post-thaw viability. Cell viability after thawing is improved compared to conventional pellet models where cells settle under gravity to form a pseudo-tissue before freezing. The technique may advance cryobiology and other applications that demand high-integrity transport of pre-assembled 3D models (from cell lines and in future cells from patients) between facilities, for example between medical practice, research and testing facilities.

1. Introduction

The term 'tissue engineering' was first used in the 1980s in the context of prosthetic devices and tissue manipulation in surgeries [1]. The true roots of tissue engineering as described today are in what is considered to be the first article in the field, titled 'Functional organ replacement: The new technology of tissue engineering' in the journal Surgical Technology International in 1991 [2]. However, cell culture finds its origins in the 1950s with the first successful attempt at culturing cells *in vitro* after harvesting them from a patient [3]. After years of constant evolution and advances, the line that separated tissue engineering and cell culture has become blurry and hard to decipher, as most of the time it is imperative to understand cell culture methods to understand the current research in tissue engineering and vice versa. Both these fields have benefited from the innovations in technology and engineering throughout the years, with major milestones such as genetic reprogramming [4], the use of biomaterials to mimic cell environments [5] or the technology to successfully cryopreserve samples [6].

Cryopreservation is defined as the process by which organs, tissues, cells, organelles and other biological cell constructs are preserved by cooling at cryogenic temperature (from −150 °C to absolute zero or −273 °C) [7]. While cryopreservation research reached its peak during the first years of the development of cell culture as a field, the advances halted after efficient protocols were established. The first reports already uncover one of the key elements to a successful cryopreservation process: the need for a cryoprotective agent (CPA) such as glycerol [8, 9]. The presence of a CPA in the freezing mixture reduces the possibility of cell damage due to water crystal formation during the freezing process [10]. In 1959, Lovelock and Bishop [11] described the first use of dimethyl sulfoxide (DMSO) as a CPA and reported higher membrane permeability compared to that of glycerol, which lead to higher viability after thawing. This work established DMSO as the golden standard for CPA in all cryopreservation

methods thereafter described. Another notable development was described by Fahy *et al* [12], where high concentrations (close to 8 molar) of DMSO could be used to create a medium devoid of ice particles at liquid nitrogen temperatures.

However, since the breakthrough of the DMSO-enabled cryopreservation and the establishment of its principles, no progress has been made to adapt the protocols to the state-of the-art in both in cell culture and tissue engineering. The present technologies allow researchers to create three-dimensional (3D) culture models [13], such as organoids [14], scaffolds [15] and bioprinted cell clusters [16], which mimic the physiological characteristics of the cells in tissue and disease. Although these 3D culture methods are up and coming, they lack optimized cryopreservation protocols. Ubiquitously used methods to cryopreserve either tissue or cell suspensions are not suitable for 3D cultures, which widens the gap between cryobiology and the current advances in tissue engineering.

While the cryopreservation of cell suspensions serves its purpose in a cell bank setting and to store cell lines for distribution and future use, it presents multiple limitations when trying to preserve the cells already settled in bioengineered systems. The cells cryopreserved in suspension lose their 3D anchors, forcing them to change their morphology and affecting their viability after thawing [17]. Furthermore, when thawing cells in suspension it is imperative to allow for a rest period between thawing and its intended experimental use. This rest period grants the cells time to acclimate to the growth medium, reach the optimal temperature for culture, secrete the toxic CPA, and reactivate cell function, all halted by the freezing process [18]. The rest period is, hence, both necessary and critical to ensure cell survival and functionality after thawing.

This time constriction forces scientists to plan and ready the cells long before the experiment is performed, having to maintain the cells in the infrastructure needed to create the ideal conditions for cell culture during this time. This limits the use of cell culture for research to those laboratories and institutes equipped to maintain the cells during this time and the period needed for preparation of the experimental setup, restricting the access to this technology to those with access to the necessary infrastructure. A cryopreservation method for 3D cell models would bridge the gap and allow researchers that do not have access to create these 3D models to obtain them and perform the necessary tests without the expertise in tissue modeling.

Moreover, when working with different cell types there is a handicap when using primary cells and attempting to store them. The functionality of primary cells decays rapidly when removed from physiological conditions [19]. When its functionality is stabilized using outside physical and chemical artificial stimuli, their viability plummets after 5–20 passages [20], making time a critical factor when creating bioengineered models for disease etiology or drug testing. The assembly of these models directly after cell harvest is imperative when aiming to maintain cell morphology, functionality, and viability [21]. The possibility of cryopreserving these models already assembled and cell-laden would drastically improve the viability and function of the cells harvested while reducing the rest period after thawing by using the already assembled 3D model.

We propose a method to cryopreserve hepatic epithelial organoids [13] in a 3D cell-laden 1% carboxymethyl cellulose scaffold that maintains cell morphology and viability. The scaffold provides structure for the hepatocytes to create spheroids on their own as well as support throughout the freezing and thawing processes for optimal cell viability post-thawing while withstanding the process and maintaining structural integrity. Furthermore, this method is set to achieve higher cell viability than transporting the disaggregated cells as a cryopreserved pellet for model assembly after thawing, allowing the cells to settle and form a tissue beforehand to improve viability after cryopreservation. This technique constitutes a step forward for it will facilitate the creation, study, and transport of already assembled 3D models from cell lines or primary cells from patients.

2. Methods

The materials for the biofabrication of the scaffolds and NMR acquisitions were obtained from Sigma-Aldrich, Germany. All cell culture reagents were obtained from Thermofisher Scientific—Gibco™ unless stated otherwise.

2.1. Cryogel scaffolds fabrication

Carboxymethyl cellulose (CMC) cryogels were fabricated as described by Velasco-Mallorquí *et al* [22]. Briefly, a crosslinker mixture containing 0.5 M 2-ethanesulfonic acid (MES) buffer at 5.5 pH, 50 mg ml^{-1} adipic acid dihydrazide (AAD), and 1 μg $μl^{-1}$ N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) was vortexed until homogeneity was achieved. The crosslinking solution was then added to the solution of 1% CMC diluted in MilliQ water at a concentration of 10 mg ml^{-1} at 45 °C. The final solution contained 1 ml of 1% CMC dilution, 50 mM of MES buffer, 1.83 mM AAD, and 18.9 μM EDC. The mixture was energetically pipetted to avoid early crosslinking upon mixing. For fluorescein-stained cryogels, 10.9 μM fluoresceinamine was added to the final solution before polymerization.

Immediately after mixing, the solution was swiftly pipetted into polydimethylsiloxane (PDMS) molds (annular cylinders, 2 mm high, 10 mm diameter) over a microscope glass slide for support. Subsequently,

the molds were covered with a cover glass and placed at −20 °C overnight. The cryogels were carefully removed from the molds, submerged in phosphate-buffered saline (PBS), and cut into 5 mm diameter discs using a 5 mm biopsy punch. The cryogels were then moved into fresh PBS and autoclaved to sterilize for cell culture use.

2.2. Cell culture and 3D cell seeding

AML12 cells (alpha mouse liver hepatocyte cell line, ATCC® CRL-2254™) were cultured in Dulbecco's modified Eagle's medium/Ham's F12 nutrient mixture (1:1)—GlutaMAX™, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% Penicillin-Streptomycin, 10 μg ml^{-1} insulin, 5.5 μg ml^{-1} transferrin, 5 ng ml^{-1} selenium and 40 ng ml^{-1} dexamethasone. The cells were incubated at 37 °C and 5% CO_2 in a humidified atmosphere. Culture media was changed every 48–72 h. Once the cells reached 70%–80% confluency and promptly before the experimental procedure, the culture vessel was washed three times with PBS. The cells were then detached from the culturing vessel using Trypsin-EDTA (0.25%) solution and centrifuged (250 g, 5 min). The supernatant was discarded and the pellet resuspended in complete growth medium at a density of 1×10^6 cells/10 μl for seeding onto the scaffolds.

The cryogels were lined up in 12 well plates for partial dehydration. After the excess of PBS was removed, the cell suspension was seeded on each scaffold, re-pipetting the excess cell mixture on top of the cryogel for improved yield. The plate was then covered and introduced in the incubator, left undisturbed without extra media for 20 min to allow for cell settlement, and finally 2 ml of fresh growth medium carefully added to each well after this period.

Twenty four hours after seeding, the cryogels were moved to a new plate with fresh growth medium. The cells left behind at the bottom of the seeding plate were counted to assess the efficiency of the seeding procedure. The cell-laden cryogels started the cryopreservation process 24 h after seeding without being moved onto a new plate.

2.3. Freezing procedure

Freezing media was prepared using fresh growth media, modifying the FBS concentration to 20% and adding 5% DMSO (v/v) as a CPA. For these experiments, 1 ml of freezing medium was added in each cryovial and two scaffolds (either cell-laden or cell-free) were gently scooped from the well into each cryovial. The cryovials were swiftly moved into a freezing container with 2-propanol for gradual temperature decrease (−1 °C per minute). The container was introduced in the −80 °C freezer overnight. The following day, the cryovials were removed from the freezing container and set in a vapor phase nitrogen tank for long term storage. The samples were maintained in the nitrogen tanks for two weeks before thawing.

2.4. Thawing procedure

The cryovials were removed from the nitrogen phase tank and quickly thawed at 37 °C using a water bath. After thawing, the contents of the cryovials were poured in a 10 cm sterile petri dish with 15 ml of warm sterile PBS. The scaffolds were gently swirled in the warmed PBS for 10 min to remove the leftover freezing media from the biomaterial. After washing, the cryogels were moved into fresh growth medium in a new 12 well plate and set in the the incubator. The growth medium was changed every 48–72 h.

2.5. Cell viability assay

Cell viability was assessed with both alamarBlue™ Cell Viability Reagent (Thermo Fisher Scientific, Spain) and confocal microscopy imaging (LSM 800, Leica).

The cell-laden scaffolds that did not undergo cryopreservation were kept in culture until day 11 after seeding. The cryopreserved cell-laden scaffolds underwent cryopreservation.

The alamarBlue assay was performed in 96 well plates, where each cell-laden scaffold was set in a well with 100 μl of 10% alamarBlue in media solution. The plates were incubated for 3 h and analyzed in a microplate reader (Infinite M200 PRO, Tecan) with 560 nm excitation and 590 nm emission filters. This assay was used to calculate metabolic activity of the cells inside the cryogel on days 2, 4, 7, 9, 11 [23].

After staining, the cell-laden scaffolds were imaged using the confocal microscope LSM 800 Leica scanning laser confocal microscope operating with the ZEN 2.3 (blue edition) imaging software to assess cell viability in the 3D constructs. The cryogels containing fluoresceinamide were used to image the fibers of the material. Live cell staining was performed to assess viability avoiding scaffold collapse by maintaining the hydrostatic pressure inside the cell-laden scaffolds. The dead cells were stained with propidium iodide (PI) (Sigma Aldrich, Germany) while Hoechst 33342 (Thermo Fisher Scientific, Spain) was used as a counterstain. The cell-laden cryogels were stained in a sterile PBS solution containing 500 nM PI and 1 mg ml^{-1} Hoechst 33324. The cryogels were incubated in the solution for 30 min before washing them with PBS to remove excess dye. After washing, the cryogels were set in the PDMS moulds mounted on a microscope slide to avoid cryogel collapse. The mould was filled with fresh PBS and covered with a coverslip for imaging.

2.6. DMSO quantification

DMSO quantification by proton NMR assessed the concentration of DMSO in the samples after thawing, both in cells frozen in suspension and in 3D cell

spheroids frozen inside the cryogels. The cell suspension was frozen following the same protocol as the one used for the 3D cell constructs. Sample preparation for NMR was performed 72 h after thawing for both sample groups, allowing the cells to acclimate to the growth media in culture conditions. Briefly, the frozen cell suspension was thawed by bringing it to 37 °C and transferred to a cell culture vessel with 9 ml of pre-warmed complete growth media. The 3D constructs were brought to 37 °C and transferred to a cell culture plate with 2 ml of pre-warmed complete growth media. After 72 h, the samples were snap-frozen with 1 ml of growth media under liquid N_2 for cell lysate. Then 700 µl of the resulting supernatant and 100 µl of D2O (deuterium oxide) were transferred into a 5 mm-o.d. NMR tube.

^{1}H-NMR spectra was acquired in aqueous solution at 298 K using a 9.4 T Bruker Avance-III HD spectrometer equipped with a cryoprobe and TopSpin 2.1 software. For ^{1}H chemical shifts reference and metabolites quantification, 10 µl of a standard of disodium trimethylsilylpropanesulfonate (DSS, Cortecnet, Paris, France) in water were added to each sample tube ($\delta_{DSS} = 0.000$ ppm; final DSS concentration = 0.114 mM). Acquisition parameters for all ^{1}H-NMR measurements were as follow: acquisition time of 2.5 s; 64 K data points; 90° flip angle; $d_1 = 15$ s and 128 scans. Solvent suppression experiments were performed with a 1D NOESY presaturation pulse sequence (noesypr1d) with the above acquisition parameters and the offset presaturation frequency at 4.71 ppm (O1d) referenced to DSS. Data were processed using MestReNova software (Mestrelab Research, v. 14.2.0).

2.7. Scaffold characterization before and after cryopreservation

Mechanical properties of the biomaterial used were previously reported by Velasco-Mallorquí *et al* [22].

The time course images of spheroid formation were obtained every hour for 16 h with an inverted fluorescent cell observer microscope (Zeiss) at 37 °C and 5% CO_2 incubation conditions.

Scanning electron microscopy (SEM) observations of cell-free scaffolds for material integrity assessment were performed with a NOVA NanoSEM 230 (FEI Company) microscope at 5.0 kV. Cell-free cryogel scaffolds were dehydrated via ethanol substitution, washing the cryogels with ethanol 50%, 70%, 80%, 90%, 96% (x2), and 99.5%. Once all the water was substituted for ethanol, ethanol was replaced by CO_2 with a critical point drying protocol. Gold sputtering was done on the samples before SEM imaging for improved resolution.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9.2.0. Statistical significance was determined by a one-way ANOVA. Results were considered significant at $p < 0.05$.

3. Results

3.1. Validation of the structural stability of the cryogel scaffolds post-cryopreservation

The validation of the structural viability of the scaffold was performed before and after cryopreservation to assess its integrity throughout the process. Figure 1 reveals that the fibers of the cryogel scaffolds prevail throughout the cryopreservation and thawing process, retaining their structure, preserving their spatial distribution, and remaining functionally viable.

3.2. Evaluation of the seeding procedure and spheroid formation

The cryogels were seeded with the hepatocytes and moved immediately under the microscope for spheroid formation imaging. The cells were recorded interacting and assembling for over 48 h, while the spheroid formation occurred between the first and 10th hour after seeding. After the spheroids were formed the structures remained anchored to the fibers of the cryogel and did not migrate further though the biomaterial.

3.3. Viability assessment

Viability of the cells housed in the cryogel samples was assessed using the alamarBlue assay. The test was performed to both cryopreserved and non-cryopreserved cell-laden scaffolds to analyze metabolic activity of the cells contained in the construct.

3.4. DMSO content in the scaffold

DMSO quantification was done with ^{1}H-NMR for rapid and accurate quantification in both cell suspensions cryopreserved and thawed and the cell-laden scaffolds cryopreserved and thawed as described above. The results portrayed in figure 4(A) show a decrease in concentration in the 3D cryopreserved samples after undergoing thawing as described in this methodology. The DMSO was not quantifiable in the 3D constructs when the samples were analyzed using ^{1}H-NMR as shown in figure 4(B).

4. Discussion

4.1. The carboxymethyl cryogels withstand the freezing and thawing process and maintain structural integrity

As shown through the experimental results, the 1% CMC cryogels fabricated can withstand the freezing and thawing process when performed as described above, showing perfect fiber integrity, and allowing the cell constructs to thrive after cryopreservation. The main trait of 1% CMC cryogels when fabricated as described above is its random pore distribution and size throughout the sample (between 10 and 150 µm

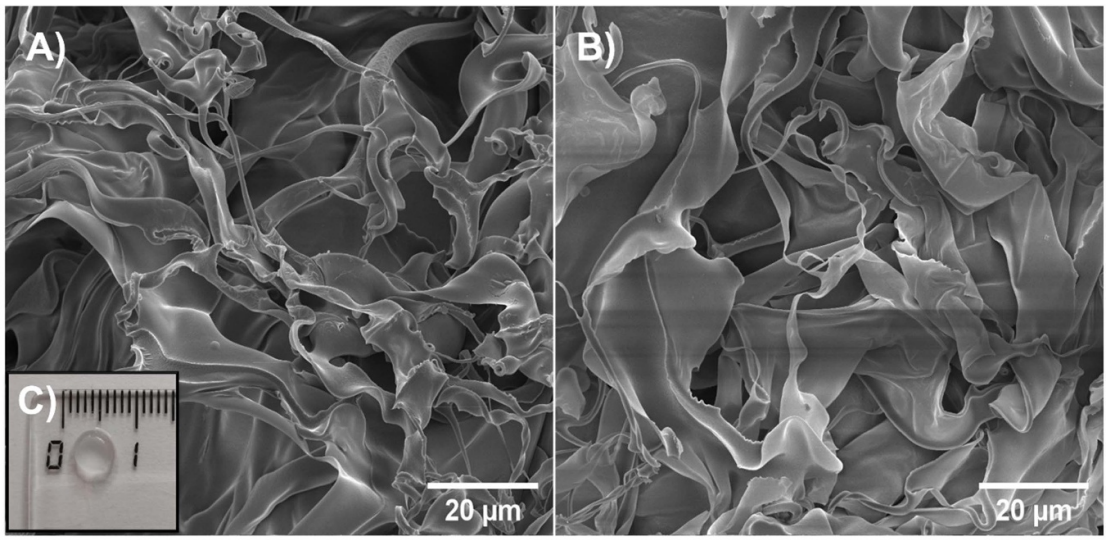

Figure 1. Fibrous structure of the CMC cell-free scaffolds imaged through SEM microscopy. (A) Structure of the dehydrated cryogels before cryopreservation. (B) Structure of the dehydrated cryogels after cryopreservation and thawing. Both structures present similarities in shape and fiber distribution and no apparent damage. (C) Gross look of the scaffold compared to a ruler showing its dimensions.

in length) [22]. These pore variability impacts cell distribution after seeding, influencing spheroid arrangement. Furthermore, this heterogenous pore distribution favors rapid diffusion of media throughout the structure, aiding in distributing the CPA-containing freezing media to all the spheroids, optimizing the freezing protocol further.

4.2. Spheroids self-assemble once the cells are seeded in the scaffold

The main advantage to working with a matrix is the possibility of reproducing physical and physiological conditions from the tissue of origin, improving fidelity of cell functions to those of the tissue being mimicked [24]. The use of the 1% CMC permits the seeding of both individual cells in suspension and already formed cell constructs [22]. Due to the inherent properties of CMC [25], the biomaterial presents enough anchors for the cells to attach and still have a higher affinity for cell-cell linkage inside the cryogels when seeded individually, promoting spheroid formation in hepatocytes (figure 2). These spheroids allow the cells to gain physiological cell-cell interactions that mimic those found *in vivo* [26]. The self arrangements of these spheroids ensure an optimal gas exchange and nutrient diffusion in the cell constructs, imperative to maintain viability in long term cultures [27].

4.3. The cells remain viable inside the scaffold both cryopreserved and non-cryopreserved up to 11 days

As shown in figure 3, there are no significant differences ($p > 0.3$) in metabolic output in the 11 days after seeding between both groups.

Moreover, these data show the high viability that this procedure yields on cryopreserved frozen 3D cell structures, comparable to the viability of those same constructs maintained in cell culture. Previous work shows viability between 60% and 80% survival of 2D cells preserved in DMSO using ubiquitous and well defined cryogenic protocols [26, 27]. The viability of the cryopreserved samples fabricated for this study is close to that of the samples not cryopreserved 11 days after seeding and thawing as shown in figure 3(A). This proves the efficiency of this particular cryopreservation method for rapid thawing of 3D cell constructs with high viability as soon as day 2 after thawing.

The imaging results support the data observed through the AlamarBlue assay, showing high viability of the cells housed inside the scaffold as well as their spatial distribution inside the pores. As shown in figure 3(B), the spheroids (blue) are located inside the pores of the cryogel (green) with high cell viability, with some apoptotic cells present in the vicinity of the cell constructs. While the dead cells were situated on the periphery of the spheroids, the cell structures remained intact throughout the days in culture both before and after cryopreservation.

These results support the hypothesis that this protocol could be adapted to cryopreserve primary cells and patient samples for clinical and research purposes. The gap that exists between cryobiology and regenerative medicine posts a handicap when using live cells due to their short viability under optimal conditions. The establishment of a robust cryopreservation protocol for samples could bridge the gap and allow for further advances in both fields while avoiding sample wastage [28, 29].

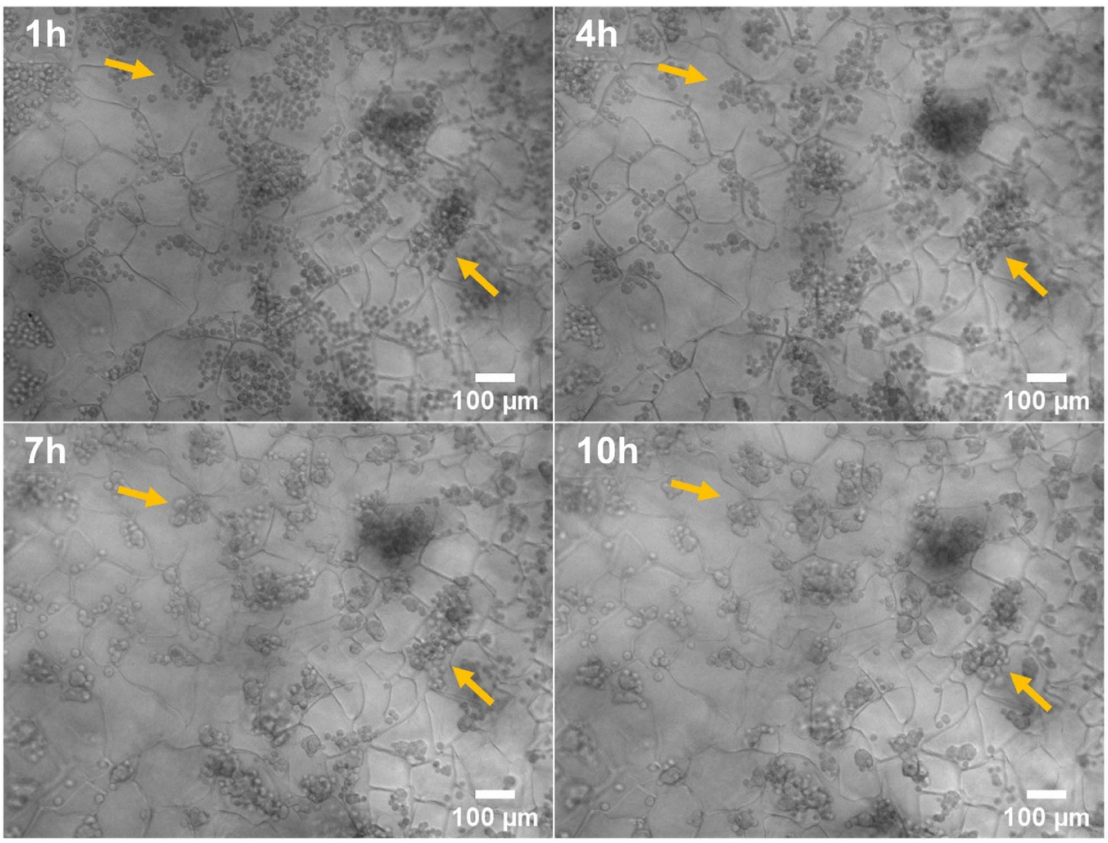

Figure 2. Time course brightfield images of spheroid formation inside the 1% CMC cell-laden scaffold taken with an optical microscope. The time elapsed from seeding is stated in each picture. Arrows point to the same representative groups of cells in each picture to guide the eye. Spheroid formation was observed starting three hours after cell seeding and up to 10 h after the event, where the cells settle into the clusters pictured on the 10 h frame. The cells self-arrange in clusters without external influence.

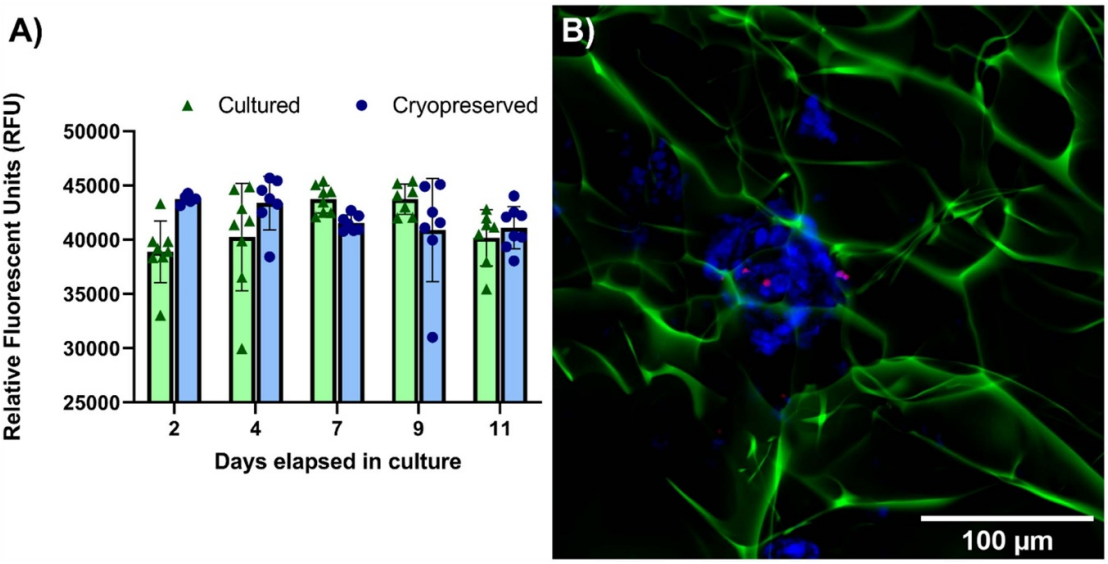

Figure 3. Cell viability of the non-cryopreserved and cryopreserved cell-laden samples. (A) Metabolic activity of the hepatocyte-laden scaffolds at days 2, 4, 7, 9 and 11 post-seeding via alamarBlue assay. Both study groups remained in culture for the same amount of time. Data are presented as mean ± SD of at least three independent experiments with three replicates each. (B) Confocal image of several cell clusters inside the cryogel 4 days after thawing. Nuclei in blue (Hoechst 33342), apoptotic cells in red (propidium iodide) and scaffold fibers in green (fluorescein).

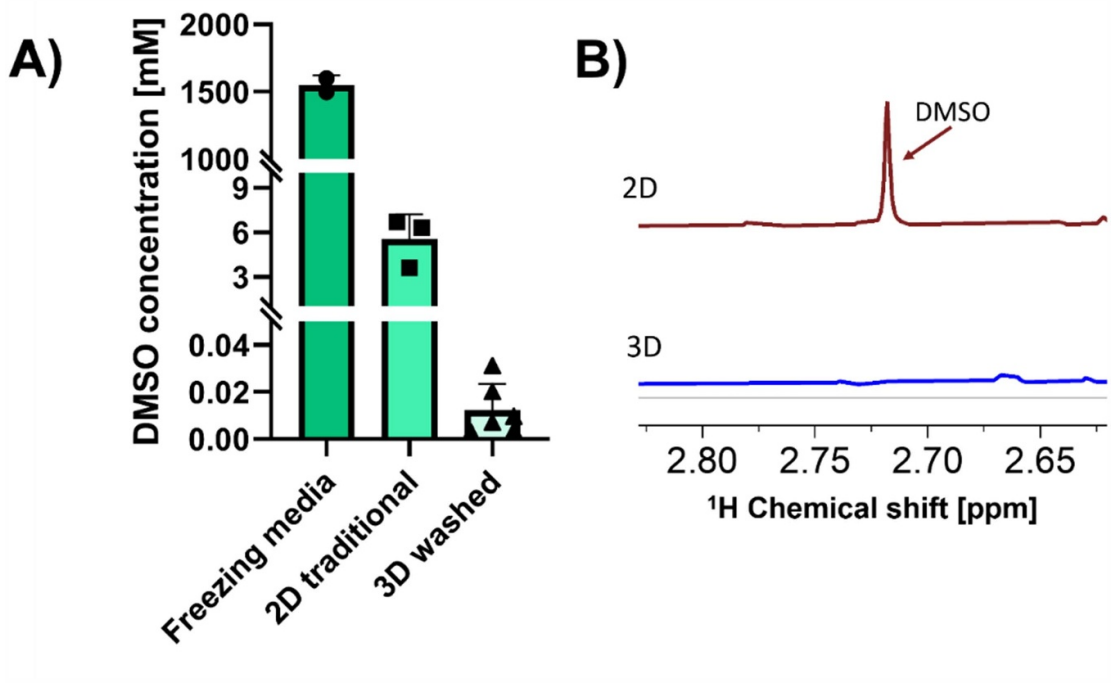

Figure 4. (A) DMSO quantification via ^{1}H-NMR in a control group (freezing media), hepatocytes frozen in suspension (2D traditional), and cell-laden scaffolds that underwent the cryopreservation protocol hereby described expressed in millimolar concentrations of DMSO. Data are presented as mean ± SD of at least two independent experiments with two replicates each. (B) ^{1}H-NMR spectrum of hepatocytes frozen in suspension (2D traditional) showing a peak identified as DMSO and ^{1}H-NMR spectrum of a cell-laden scaffold that underwent the cryopreservation protocol hereby described without DMSO peak detected.

The mechanism why the formation of cell aggregates enhances viability after thawing will be the subject of future work.

4.4. The bioengineered structures allow for CPA removal from the samples

Although DMSO has a cryoprotective effect on the cells, a long exposure to this agent drastically changes cell functionality and induces epigenetic changes due to its toxicity [30]. CPA removal after thawing is, therefore, critical for cell viability, making it imperative to check for CPA accumulation in the biomaterials and cell structures in the cryogels.

As shown in figure 4, the cryogels that were thawed and washed following our proposed protocol showed significantly lower levels of DMSO both contained inside the cell-laden cryogels and the cells when compared to the DMSO levels found in the cells frozen in suspension. These data reveal that the cell-laden scaffolds do not retain DMSO in the fibers and provide support to the cells through the washing process, allowing to significantly reduce intracellular DMSO concentration afterwards. These findings explain the high viability after the thawing process, by critically reducing the cytotoxic CPA concentrations from the sample after thawing. This points to the advantage to using both the 1% CMC cryogels and the thawing protocol proposed here when it comes to cryopreserving these hepatocellular 3D cell constructs.

5. Conclusions

In summary, we developed a cryopreservation protocol for 3D cell constructs to bridge the gap between the fields of tissue engineering and cryobiology. The current results are proof for the concept that cryopreservation of 3D cellular constructs is possible with high cell viability. The use of the 1% CMC cryogel scaffold is crucial to procure support for the cells through the process as well as to aid in the removal of the CPA after thawing for improved cell viability. The methods described here lay the ground for the development of protocols for the cryopreservation of multiple types of 3D cell constructs to extend, simplify and reduce the cost and infrastructures required for 3D cell cultures.

Data availability statement

The data generated and/or analysed during the current study are not publicly available for legal/ethical reasons but are available from the corresponding author on reasonable request.

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Conflicts of interest

IBEC has filed a patent application related to this work. A H G, M A R and I M R are co-inventors of this patent application. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this manuscript.

Author contributions

A H G co-designed and performed the cellular, cryogenic, and imaging experiments, data acquisition and processing, and co-wrote the manuscript. M A R performed the NMR experiments, analyzed the data, and reviewed the manuscript. I M R initiated the project, co-designed the experiments, co-wrote the manuscript, raised funding and supervised the work.

ORCID iDs

Alba Herrero-Gómez https://orcid.org/0000-0002-8807-0649
Marc Azagra https://orcid.org/0000-0002-5732-5303
Irene Marco-Rius https://orcid.org/0000-0001-5076-8526

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