Cellular and Molecular Bioengineering, Vol. 15, No. 5, October 2022 *(© 2022)* pp. 425–437
https://doi.org/10.1007/s12195-022-00739-7

S.I. : 2022 CMBE Young Innovators

Development of Serum-Free Media for Cryopreservation of Hydrogel Encapsulated Cell-Based Therapeutics

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(Received 17 February 2022; accepted 22 August 2022; published online 29 September 2022)

Associate Editor Michael R. King oversaw the review of this article.

Abstract

Introduction—While hydrogel encapsulation of cells has been developed to treat multiple diseases, methods to cryopreserve and maintain the composite function of therapeutic encapsulated cell products are still needed to facilitate their storage and distribution. While methods to preserve encapsulated cells, and post-synthesis have received recent attention, effective preservation mediums have not been fully defined.

Methods—We employed a two-tiered screen of an initial library of 32 different cryopreservation agent (CPA) formulations composed of different cell-permeable and impermeable agents. Formulations were assayed using dark field microscopy to evaluate alginate hydrogel matrix integrity, followed by cell viability analyses and measurements of functional secretion activity.

Results—The structural integrity of large > 1 mm alginate capsules were highly sensitive to freezing and thawing in media alone but could be recovered by a number of CPA formulations containing different cell-permeable and impermeable agents. Subsequent viability screens identified two top-performing CPA formulations that maximized capsule integrity and cell viability after storage at − 80 °C. The top formulation (10% Dimethyl sulfoxide (DMSO) and 0.3 M glucose) was demonstrated to preserve hydrogel integrity and retain cell viability beyond a critical USA FDA set 70% viability threshold while maintaining protein secretion and resultant cell potency.

Conclusions—This prioritized screen identified a cryopreservation solution that maintains the integrity of large alginate capsules and yields high viabilities and potency. Importantly, this formulation is serum-free, non-toxic, and can support the development of clinically translatable encapsulated cell-based therapeutics.

Keywords—Cell-based therapeutics, Cryopreservation, Hydrogels, Serum-free media, Mesenchymal stem cells, HUVEC, ARPE-19.

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This article is part of the CMBE 2022 Young Innovators special issue.

INTRODUCTION

A wide range of cell therapies have been developed for applications in tissue engineering, diabetes research, and cancer studies.[13,20,29] Many of these therapies employ a semi-permeable matrix, such as hydrogels like gelatin, hyaluronic acid, alginate, or collagen, to encapsulate therapeutic cells, as a way to improve treatment efficacy and extend the longevity of their therapeutic function.[4,9] The hydrogel matrix permits mass transport of nutrients, provides immune protection, creates a tissue-like environment, and immobilizes the therapeutic cells at the target site, thus increasing the site-specificity and long-term efficacy of the treatment.[14,22] These benefits have helped advance encapsulated cell-based therapeutics into clinical and commercial applications. Yet, the ability to cryopreserve and store hydrogel encapsulated cell products after their manufacture and before their delivery to patients is essential to their widespread use.[12,24] Unfortunately, storage conditions that maintain hydrogel matrix function and encapsulated cell viability have not been fully defined, which increases costs, confounds logistics, and limits commercial scalability.

Exposure to cryogenic temperatures is widely known to be detrimental to cell viability, cause abnormal therapeutic protein secretion, compromise cell surface receptor expression, and hamper cell therapeutic functions.[3] These adverse effects often result from mechanical stresses induced by ice nucleation and growth during the cooling process or recrystallization during the warming process, which can cause mechanical damage intra- and extra-cellularly.[5] Different cryopreservation agents (CPAs) have therefore been developed to manage ice crystal formation and protect cells during the freeze–thaw process. Dimethyl sulfoxide (DMSO) and glycerol are among the most widely used cell-permeable CPAs.[19,27] They can decrease a medium's freezing point and reduce intracellular ice formation and enhance dehydration.[12,13,19] These CPAs are especially needed for nucleated cells since their penetration is required to prevent intracellular ice formation and preserve their nuclear envelope. Cell impermeable mono- and disaccharides, as well as polyol agents, have also been shown to preserve cell viability and function. These CPAs are also believed to reduce ice crystal growth and inhibit ice recrystallization during the thawing process[34], presumably *via* the formation of a concentrated solute layer that prevents crystal formation outside the cell membrane.[28,35] Yet, despite these protective benefits, CPAs are also widely known to impair cell viability and function, particularly when used at high concentrations, since they can be toxic and/or introduce appreciable osmotic stress. Efforts to optimize cryopreservation methods therefore often focus on minimizing the concentrations of CPAs that are required to adequately preserve cells.

Cell encapsulation has also been explored as a means of cryopreserving cells.[11,36] Hydrogels composed of alginates have been shown to assist in cryopreservation in some settings,[6,8,15,25,31] presumably due to their porous structure which can confine ice crystal growth and alter osmotic pressures and shock.[36] For example, alginate hydrogels were found to facilitate stress resistance during the cryopreservation of encapsulated *E. coli,* thus yielding higher bacteria viability post-recovery.[11] Alginate encapsulation has also increased mouse embryonic stem cell and human adipose-derived stem cell viability during the low CPA vitrification cryopreservation process.[36] Alginate and other natural and synthetic biopolymers have been explored for other cell constructs as well.[30] However, most studies have largely focused on preserving cell viability. The impact of CPAs and associated preservation protocol on the structural integrity of the hydrogel matrix function has not been fully explored.

Based on previous findings of effective CPA compositions in freezing free cells, we developed a screening process to identify an optimized CPA formulation to cryopreserve nucleated cells encapsulated in alginate hydrogels to address the need for cryopreservation of encapsulated cell therapeutics. We prioritized solutions that can preserve $> 70\%$ cell viability (per FDA guidelines, Docket number: FDA-2008-D-0206) and capsule integrity at $-80\ ^{\circ}C$ since storage at this temperature is supported by equipment that is readily available at cGMP biologics manufacturing facilities (United States Food and Drug Administration (FDA) code, 21 CFR 600, 610, Good Manufacturing Practices (cGMP) and 21 CFR Part 1271, Good Tissue Practices (cGTP)), at hospitals, and other distribution channels for cell-based therapeutics. A list of 32 preservation media was developed. Each composition contained one cell-permeable CPA (DMSO or glycerol) and one cell-impermeable CPA (glucose, sucrose, trehalose, or PEG).[7,16–18,26,29] The preservation media were used to cryopreserve multiple mammalian cell types including native retinal pigment epithelial (RPE) cells, genetically-engineered RPE cells, human umbilical cord endothelial cells (HUVECs), and mesenchymal stem cells (MSCs) subsequent to encapsulation in alginate and cryopreservation at $-80\ ^{\circ}C$. Further, since larger capsule require greater penetration of CPA agents to preserve cell viability in the core of the capsule, we chose a large, but clinically relevant, capsule size (> 1 mm diameter) to ensure that the preservation media selected here would be appropriate as a universal cryopreservative.[32] Analyses of capsule integrity and encapsulated cell viability show that the capsules, and the cells within them, are both highly sensitive to the stresses imposed on the composite system during the freezing and thawing pro-

cess. Nevertheless, using initial microscopy-based screens of alginate capsule structure, followed by viability analyses of free and alginate encapsulated cells, we demonstrate that a CPA formulation composed of 10% DMSO and 0.3 M glucose can preserve the integrity of alginate capsules, the viability of their cells (> 70%), and their functional capacity to produce therapeutic biomacromolecules. These attributes were also retained using native and engineered RPEs, HUVECs, and MSCs, indicating the 10% DMSO and 0.3 M glucose may have broad utility for preserving multiple alginate encapsulated cell-based therapeutics.

RESULTS

Screening Preservation Media for the Maintenance of Hydrogel Integrity

The criterion for selection of a clinically translatable CPA formulation includes maintenance of hydrogel matrix integrity to preserve pore size and diffusion kinetics as well as viability and functionality of the encapsulated cells during the freezing–thawing process (Fig. 1a). For this reason, we incorporated cell-permeable and cell impermeable CPAs to maximize both of these capabilities. CPAs were combined at various concentrations to form 32 rationally designed preservation media (Fig. 1b). Figure 2a demonstrates our naming convention for CPAs, which are designated based on the identity and concentration of the cell-permeable and impermeable agents in the formulation. For example, 10D.3G contains 10% DMSO and 0.3 M glucose.

We first evaluated the ability of CPA formulations to maintain alginate capsule integrity after cryopreservation. Empty capsules of 1.43 ± 0.10 mm diameter (Supplement 1) were frozen at − 80 °C using a gradient cooling rate of 1 °C/min. One week later, the capsules were thawed rapidly in a 37 °C water bath in groups of 8 samples to ensure rapid removal of CPA formulations from the capsules. Complete media was then added, and the capsules were left for 24 h to facilitate recovery from the thawing process. Capsules were then analyzed using dark-field imaging. Prior to being frozen, the capsules were highly spherical and displayed continuous, smooth boundaries under dark-field imaging (Fig. 2b, *left panels*). Capsules that were frozen in DMEM media (M) without any CPA components displayed both shape deformation and surface damage, as demonstrated by the discontinuous boundary and bright pixels inside the boundary (Fig. 2b, *right panels*). In contrast, capsules that were frozen in CPA formulations were able to retain the hydrogel integrity, with only minor shape deformations (Fig. 2b, *middle panels*, Supplement 2–3). These data highlight that preservation media maintain alginate capsules' shape and integrity during cryopreservation, making them potential candidates for protecting cell encapsulated hydrogel capsules.

To further evaluate the structure of capsules, we analyzed the circularity and roundness of empty capsules before and after cryopreservation. Circularity captures local irregularities on the capsule boundary, while roundness measures the shape of the global capsule (Supplement 4). Since the capsules were transparent, 3D features were better captured on the 2D images by differentiating between the inner and outer boundaries of the capsules (Supplement 5). Analyses of dark-field images revealed that capsule circularity varied more significantly across preservation media formulations than roundness. Among all cell non-permeable CPAs, trehalose-based media yielded the highest capsule circularity, demonstrating its enhanced performance in protecting hydrogel integrity.

We next calculated a performance score based on the quantification of the circularity and roundness of each capsule's inner and outer boundaries for each CPA formulation (Fig. 2c). A higher score indicated better capsule protection. Binary image intensity inside each capsule boundary was determined to eliminate preservation media in which the capsules experience severe surface damage. The top 7 CPAs identified with this screen most commonly contained DMSO as the cell-permeable and trehalose as the cell impermeable agent. Nevertheless, formulations with glycerol, glucose, and polyethylene glycol were also shown to improve the integrity performance scores. We therefore progressed formulations that contained all of these agents for viability testing.

Identification of Capsule-Integrity-Preserving Formulations that Also Maintain Cell Viability

Next, we selected 5 of the top 7 CPA formulations that maintained capsule integrity and assayed them for the ability to preserve the viability of free (unencapsulated) cells during cryopreservation (Fig. 2c). Each CPA formulation was used to freeze RPE cells for 24 h at − 80 °C. After thawing, cell viability was assayed for each sample *via* trypan blue staining. Cells that were cryopreserved using any of the top CPA formulations displayed greater than 85% viability, and, thus, surpassed the FDA stipulated requirement of greater than 70% viability (Fig. 2d). In contrast, cells that were frozen in media that did not contain CPAs had significantly lower viability of only 62% and thus did not meet the requirement. Two CPA formulations, 10% DMSO with 0.3 M trehalose (10D.3T) and 10% DMSO with 0.3 M glucose (10D.3G) greatly exceeded this benchmark and yielded the top two post thaw cell viabilities of 93.6 and 93.1%, respectively.

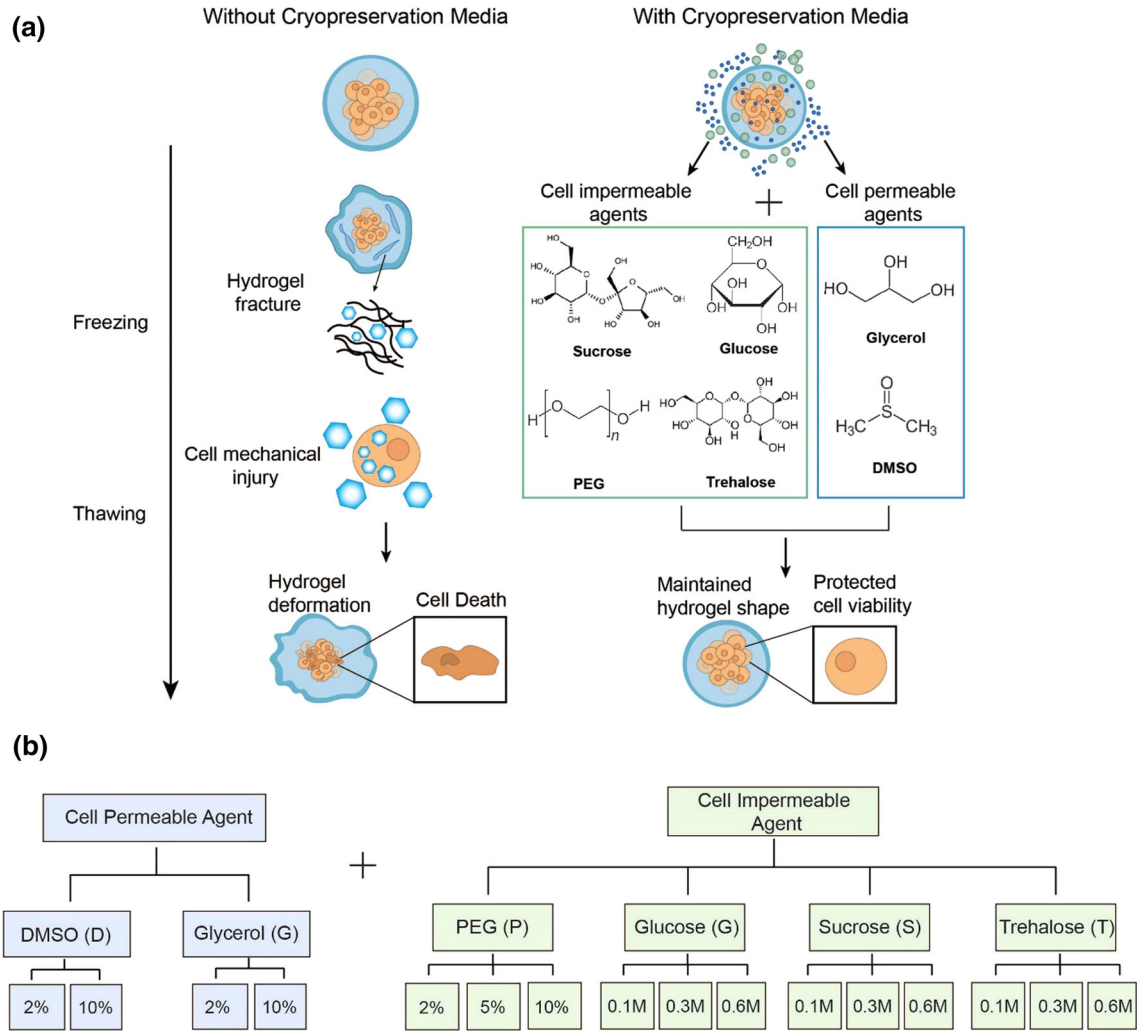


FIGURE 1. CPA protects hydrogel integrity and viability. (a) Schematic of the mechanisms of cell-permeable and cell-impermeable CPAs during cryopreservation. (b) Chart outlining the choices of cell-permeable and cell-impermeable agents explored in this manuscript.

Verification that the Top CPA Formulations Preserve Encapsulated Cell Viability After Cryopreservation

We next examined the ability of the top two CPA formulations from the capsule integrity and cell viability screens (10D.3T and 10D.3G) to preserve the viability of encapsulated cells after cryopreservation. We also included a third top CPA formulation that contained glycerol in place of DMSO (10G.3T). All three CPA formulations were compared to media alone (M). Alginate capsules containing RPE cells were fabricated using a microfluidic device[21] and incubated in either 10D.3T, 10D.3G, 10G.3T, or M at room temperature for 15 min. The encapsulated cells were then frozen at − 80 °C and thawed using the same procedure employed for the free cells described above (8 samples at a time).

Consistent with the structural analyses in Fig. 2, all three CPA formulations were found to preserve the capsule integrity upon inspection with dark-field microscopy (Fig. 3a). Only the capsules frozen in M displayed structural deformations. Cell viability was assayed after gently lysing the hydrogel matrix of the capsules using alginate lyase to release the cells and facilitate a trypan blue staining assay. We observed no change in the number of cells recovered from the capsules following lysis (Fig. 3b). The viability of encapsulated cells frozen in M was only 7.1 ± 0.5% (Fig. 3c) which is nearly 6 times lower than that of free cells frozen in M alone. Importantly, however, viability was restored appreciably using 10D.3G, 10D.3T, or 10G.3T (84.0 ± 2.3, 71.8 ± 4.3, or 57.6 ± 5.1%, respectively), but only 10D.3G and 10D.3T surpassed

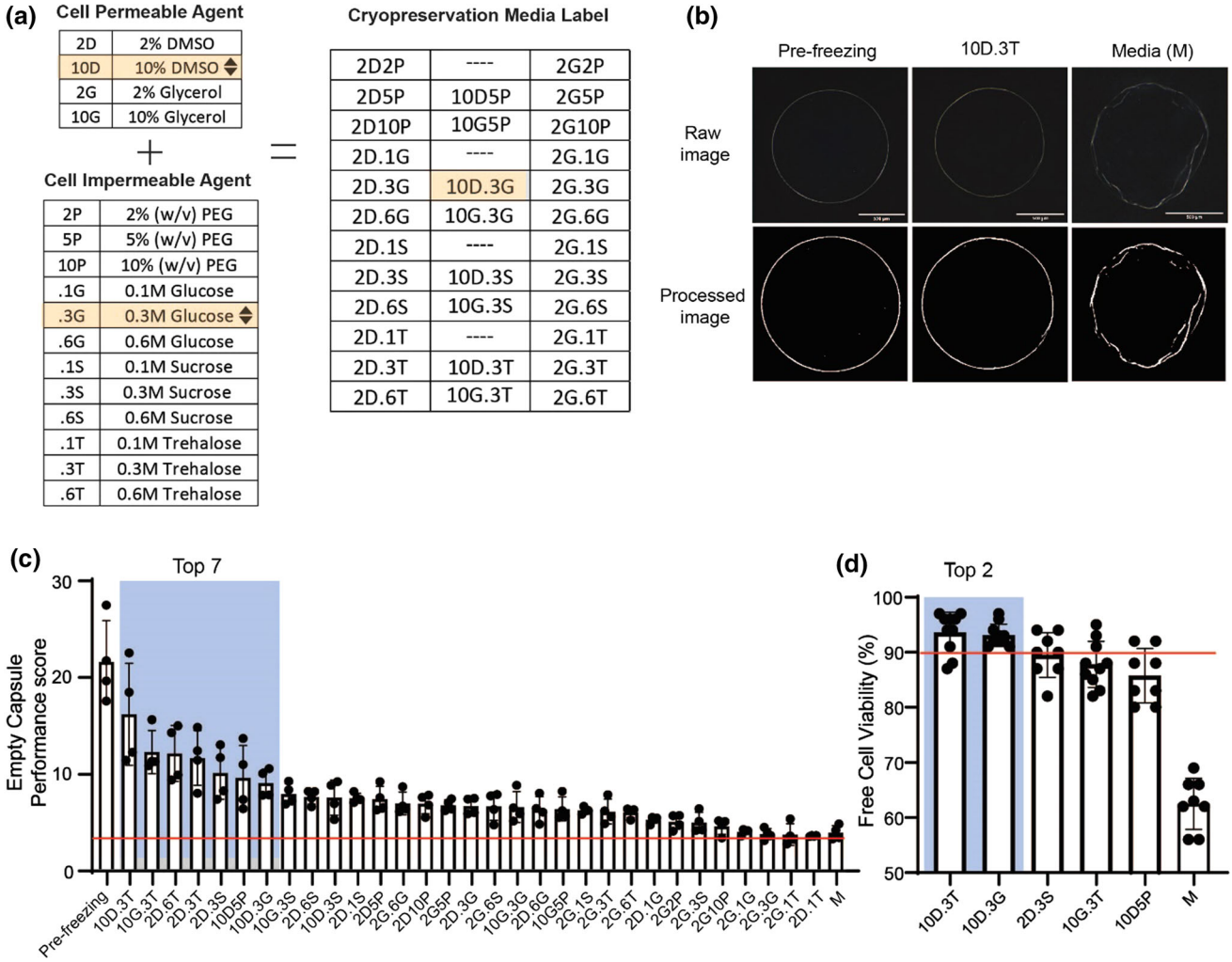


FIGURE 2. Screening of preservation media that protects capsule integrity and free cell viability during cryopreservation at − 80. (a) Chart demonstrating the naming convention for CPAs evaluated in this manuscript. For example, 10D.3G contains 10% DMSO and 0.3 M glucose. (b) Raw darkfield image (top row) and binary converted image (bottom row) of capsules before freezing (pre-freezing), 10D.3T, and media only (M) after one-week cryopreservation. The scale bar represents 500 μm. (c) Performance score for each cryopreservation media calculated based on capsule circularity and roundness measurements. A total of 4–5 capsules in each condition were scored. The data shown here are representative of two individual experiments. Scores were ranked from high to low. The highest seven CPAs were highlighted in the blue box. The red line indicates the performance score of media only (M) condition. (d) Free cell viability for each selected CPA formulation. The data shown here are representative of two individual experiments. Scores were ranked from high to low. The highest two CPA formulations were highlighted in the blue box. Red line indicates 90% cell viability post thawing. A total of 7–8 samples in each condition were scored.

the FDA benchmark for the viability of greater than 70% (Fig. 3c).

The viability results with trypan blue staining were also further confirmed through visualization and quantification of floating/dead cells 24 h after being plated in a 6-well plate. Consistent with the trypan blue assay, wells from the M group contained significantly more floating cells than the other groups (Fig. 3d). Moreover, cryopreservation using 10G.3T had significantly more floating cells than 10D.3G or 10D.3T (2.2x and 2.8x, respectively), indicating DMSO may perform better than glycerol as the cell-permeable agent for preserving encapsulated RPE cells. Analyses of cell adherence mirrored this effect, with the 10D.3G and 10D.3T preserved capsules displaying appreciably more adherent cells than experiments with 10G.3T (Fig. 3e). The M group exhibited little to no cell adherence.

Glucose and Trehalose-Based Media Preserve RPE Cell Viability and Protein Secretion Activity

To explore the impact of our two top-performing CPA formulations (10D.3G and 10D.3T) on RPE cell function, we next examined their influence on the secretion capacities of encapsulated RPE cells that

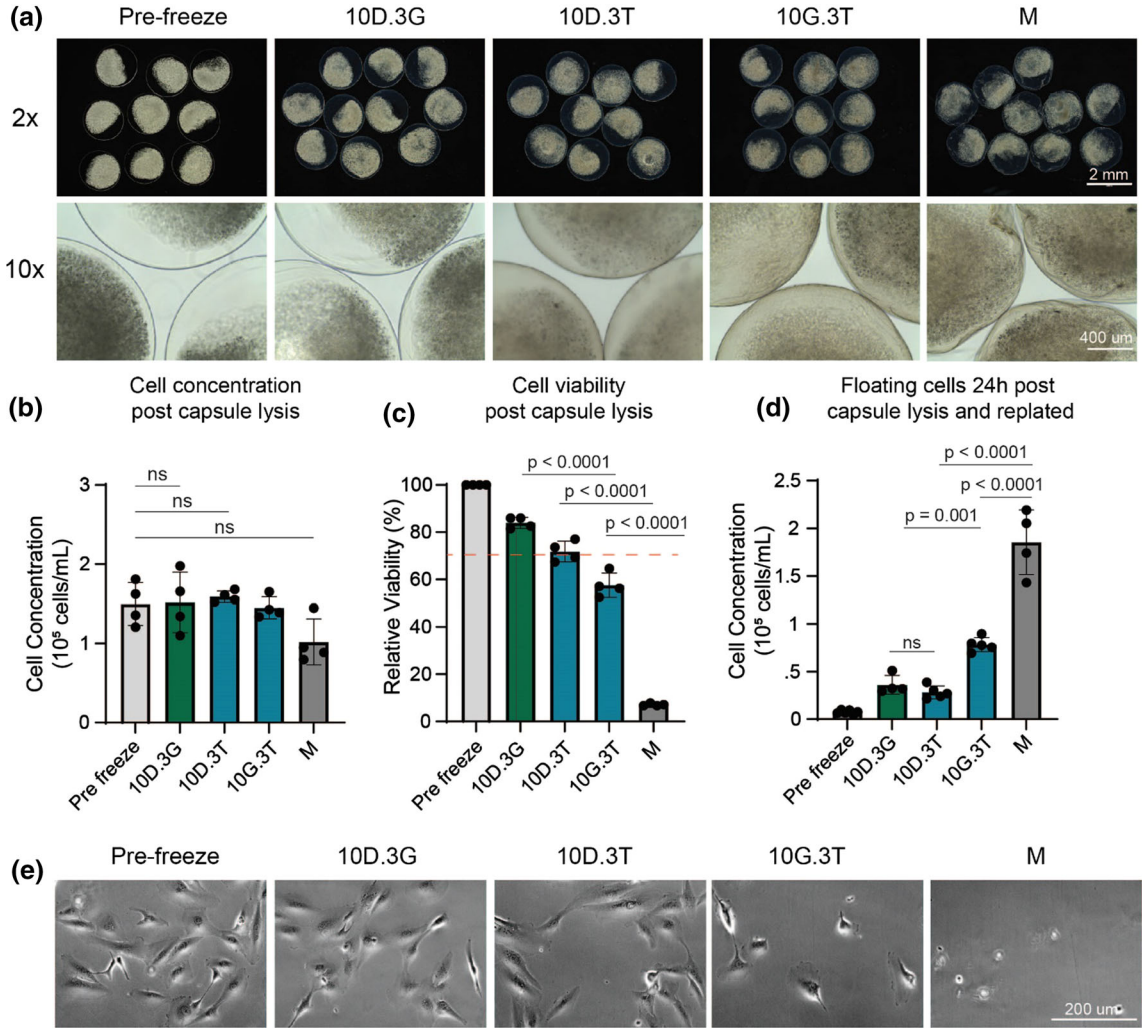
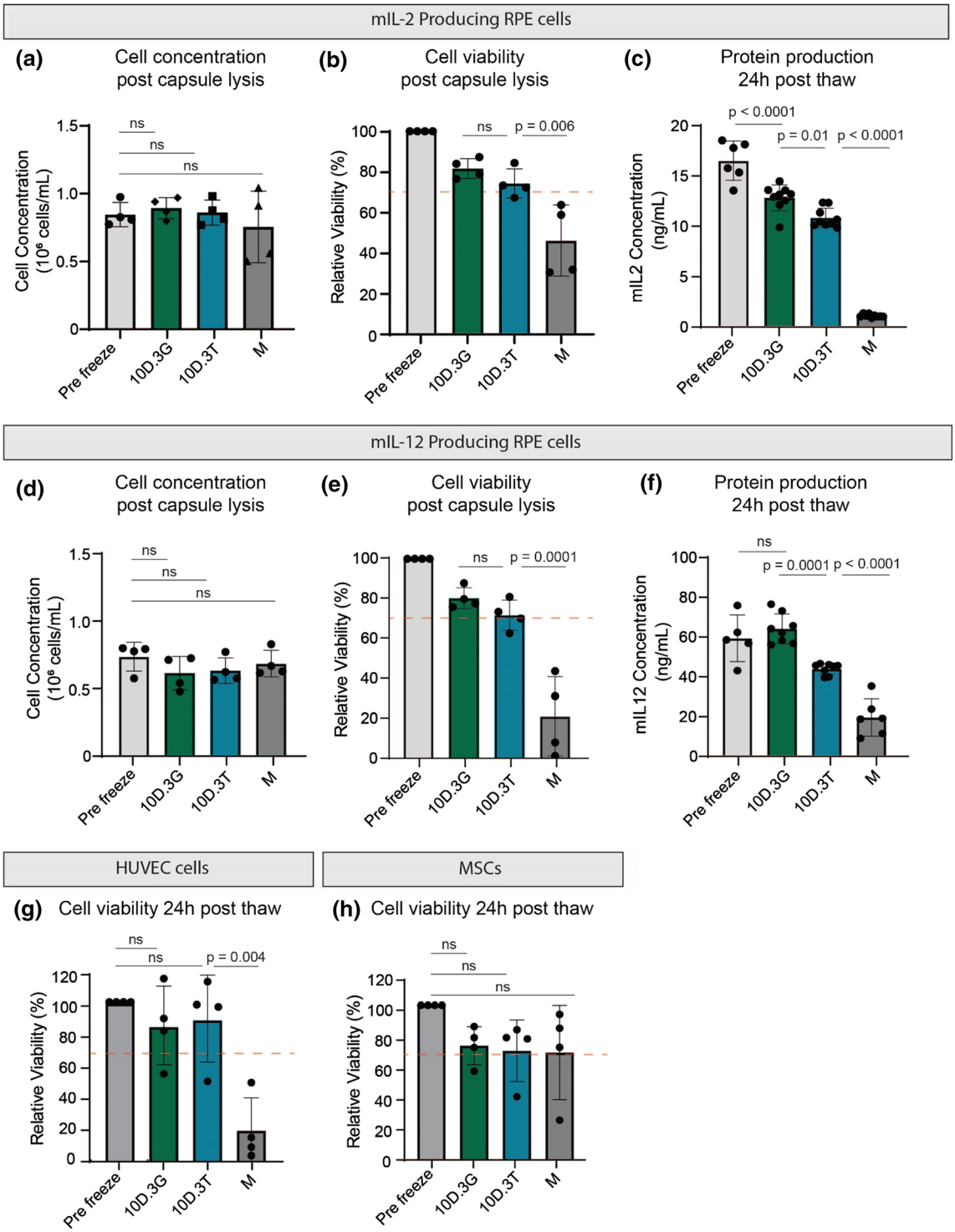


FIGURE 3. Preservation media protects cell viability during cryopreservation. (a) Darkfield image of RPE capsules pre-freeze or post thaw in various CPA formulations. 2x magnification, scale bar indicates 2 mm (top). 10x magnification, scale bar indicates 400 μm (bottom). (b) Cell concentration post capsule lysis following capsule freezing and thawing in various CPA formulations. Each data point represents 10 capsules lysed. (c) Relative cell viability post capsule lysis following capsule freezing and thawing in various CPA formulations. Each data point represents 10 capsules lysed. Red dashed line represents 70% viability threshold. (d) Concentration of cells floating in cell media 24 h after being plated in a 6-well plate. Each data point represents an individual well. (e) Darkfield imaging of cells 24 h after being plated in a 6-well plate. 20x magnification, scale bar indicates 200 μm. *P* values were acquired using one way ANOVA with Holm-Sidak method for multiple comparisons, ns indicates not significant. Each image is representative of four wells imaged. All plots shown here are representative of three individual experiments.

were engineered to produce the cytokines interleukin-2 (IL-2) or interleukin-12 (IL-12). Nash *et al.* have previously demonstrated that genetically engineered RPE cells that produce these cytokines have significant therapeutic potential in cancer immunotherapy.[21] RPE-mIL2 and RPE-mIL12 encapsulated capsules were fabricated, frozen, stored for 24 h, thawed, and lysed to assess viability as described above. The number of cells recovered post thaw and lysis for each engineered cell line remained similar across CPA formulation, media, and control (never frozen) groups (Figs. 4a, 4d). Moreover, as with the native RPEs, cell viabilities using 10D.3G or 10D.3T were found to meet or exceeded the 70% benchmark using both engineered cell lines (Figs. 4b, 4e). Further, the 10D.3G formulation was also found to improve cytokine production. Cryopreservation with this formulation resulted in only a small decrease in IL-2 secretion and yielded statistically indistinguishable IL-12 titers compared to encapsulated cells that were not subjected to freezing


mIL-2 Producing RPE cells
(a) Cell concentration post capsule lysis
Cell Concentration (10⁶ cells/mL)
ns
ns
ns
(b) Cell viability post capsule lysis
Relative Viability (%)
ns
p = 0.006
(c) Protein production 24h post thaw
mIL2 Concentration (ng/mL)
p < 0.0001
p = 0.01
p < 0.0001
Pre freeze
10D.3G
10D.3T
M
mIL-12 Producing RPE cells
(d) Cell concentration post capsule lysis
Cell Concentration (10⁶ cells/mL)
(e) Cell viability post capsule lysis
Relative Viability (%)
ns
p = 0.0001
(f) Protein production 24h post thaw
mIL12 Concentration (ng/mL)
ns
p = 0.0001
p < 0.0001
HUVEC cells
(g) Cell viability 24h post thaw
Relative Viability (%)
ns
ns
p = 0.004
MSCs
(h) Cell viability 24h post thaw
Relative Viability (%)
ns
ns
ns

◀**FIGURE 4. Preservation media give universal protection of cell viability. (a) Cell concentration post capsule lysis following capsule freezing and thawing either before freezing or 24 h post thawing in 10D.3T, 10D.3G, or media only (M). Each data point represents 10 capsules lysed. (b) Relative cell viability post capsule lysis following capsule freezing and thawing before freezing or 24 h post thawing in 10D.3T, 10D.3G, or media only (M). Each data point represents 10 capsules lysed. Red dashed line represents 70% viability threshold. (c) mIL-2 concentration from individual capsules before freezing or 24 h post thawing in 10D.3T, 10D.3G, or media only (M). (d) Cell concentration post capsule lysis following capsule freezing and thawing before freezing or 24 h post thawing in 10D.3T, 10D.3G, or media only (M). Each data point represents 10 capsules lysed. (e) Relative cell viability post capsule lysis following capsule freezing and thawing in various CPA. Each data point represents 10 capsules lysed. Red dashed line represents 70% viability threshold. (f) mIL-12 concentration from individual capsules before freezing or 24 h post thawing in 10D.3T, 10D.3G, or media only (M). (g) Relative HUVEC viability post capsule lysis following capsule freezing and thawing before freezing or 24 h post thawing in 10D.3T, 10D.3G, or media only (M). Each data point represents an individual capsule. Red dashed line represents 70% viability threshold. (h) Relative MSC viability post capsule lysis following capsule freezing and thawing before freezing or 24 h post thawing in 10D.3T, 10D.3G, or media only (M). Each data point represents an individual capsule. Red dashed line represents 70% viability threshold. P values were acquired using one way ANOVA with Holm-Sidak method for multiple comparisons, ns indicates not significant.**

(Figs. 4c, 4f). Production of IL-2 and IL-12 decreased after cryopreservation in the 10D.3T CPA formulation, indicating that inclusion of glucose as the impermeable component aided in preserving protein secretion function.

Glucose and Trehalose-Based Media Maintain Cell Viability with Multiple Cell Lines

To further test the broad utility of the 10D.3G and 10D.3T CPA formulations, we also evaluated their ability to cryoprotect other cell types in addition RPEs. Our analyses focused on human umbilical vein endothelial cells (HUVECs) and mesenchymal stem cells (MSCs) since both of these cells are widely used in cell therapies to treat many diseases including diabetes, cancer, and cardiac diseases.[13,20,29] Both cell types were encapsulated in alginate as described above and stored for 1 week. We developed a secondary viability assay technique for these cells that were too fragile to withstand the capsule lysing process. This method was carefully validated alongside the lysing method we described above and is described in detail in the methods sections and Supplement 5. Each cell line was thawed using the method described above. Similar to the RPE cells, the viability of encapsulated HUVECs and MSCs did not vary appreciably between the two CPA formulations and were both above the 70% FDA benchmark (Figs. 4g, 4h). Unexpectedly, MSCs exhibited near 70% viability using media alone, indicating a different sensitivity towards cryopreservation when encapsulated.

The Lead CPA Formulation Penetrates Large 1.5 mm Capsules Providing Spatially Uniform Protection

Finally, to verify cell viability within intact capsules, samples that were frozen and thawed in 10D.3G were subjected to live/dead staining (calcein am/ethidium homodimer-1) and imaging using 2D and 3D confocal microscopy (Figs. 5a, 5b). The 10D.3G capsules showed primarily live cells and appeared most similar to encapsulated cells that were never frozen. Further, capsules frozen in this CPA formulation were cryopreserved for two weeks and remained above the FDA benchmark after being thawed (Fig. 5c). Taken together, the data presented in this work demonstrate that 10D.3G or 10D.3T demonstrated distinct ability to preserve hydrogel integrity and viability of encapsulated cells, rising as the best candidates for the preservation of encapsulated cells.

DISCUSSION

Cell encapsulation technology enables the delivery of allogeneic or xenogeneic cells as therapeutic agents within an immune-isolating membrane, which can overcome challenges of long-term local and systemically controlled therapeutic release and organ graft rejections.[1,2,4] With the development of these encapsulated cell therapies in treating versatile diseases, corresponding solutions to preserve them become essential for clinical translation and commercialization.[10,14,23,33] To support cryopreservation, CPA formulations must be able to maintain the structural integrity of the hydrogel matrix in addition to the viability and therapeutic function of the encapsulated cells after thawing. This control is essential to minimize cell death and unwanted immune reactions due to the loss of the protective shell surrounding the encapsulated cells.

We prioritized CPA formulations that exhibited the ability to maintain alginate capsule integrity in an initial screen of 32 CPA formulations containing cell-permeable and impermeable agents. Virtually all of the 32 CPA formulations improved the preservation of capsules over the use of media alone, with 7 top candidates yielding appreciably higher performance. While most of these formulations contained DMSO as the cell-permeable agent and trehalose as the impermeable agent, glucose, sucrose, and polyethylene glycol (PEG) were also included in this top group. This result suggests a range of CPA formulations, and their

associated mechanisms that influence ice crystal formation during freezing and thaw can likely be used to preserve alginate-based capsules.

Consistent with the preservation of cell viability, both 10D.3T and 10D.3G also demonstrated the ability to retain the secretion functions of engineered RPEs, with 10D.3G emerging as the top CPA formulation in this category. Among other effects, this could potentially stem from a beneficial influence of glucose concentration on metabolic energy utilization and protein production. Similar benefits were demonstrated using HUVEC cells, which also exhibited high viabilities after preservation in 10D.3G or 10D.3T. While this indicates these CPA formulations likely have broader utility for cryopreservation, it was unclear whether the addition of 10D.3G or 10D.3T helped preserve MSCs, another key type of therapeutic cell. In this case, both CPA formulations performed similarly to media with respect to cell viability, which nearly met the 70% FDA viability recommendation on its own. Nevertheless, these two CPAs would still be beneficial to encapsulated MCS preservation considering their ability to, again, maintain capsule integrity and protective barrier function for the cells. Moreover, while further optimization of MSC viability is likely possible with a dedicated screen for these cells, an improved MSC-specific formulation would still need to address the maintenance of the hydrogel matrix. We anticipate efforts to identify such cell-specific formulations would benefit from our prioritized screening approach.

METHODS

Capsule Production

SLG20 alginate (PROVONA, 4202001) was dissolved at 1.4%(w/v) in saline by stirring overnight. The alginate solution was filled into both the core and shell nozzle. The core flow rate was 5 ml/h while the shell flow rate was 6 ml/h. Voltage of 5.6 kV was added between the nozzle and crosslinking bath to produce capsules that were about 1.5 mm in diameter. The capsules were crosslinked for 15 min and then washed with HEPES buffer and DMEM base media before cryopreservation.

Cell Engineering and Encapsulation

RPE cells were engineered to express mIL-2 or mIL-12 according to previous report.[21] MSCs were purchased from ATCC (SCRC-4000) and cultured in DMEM from Thermo Fisher (10564011) with 10% Fetal Bovine Serum (A3840001) and 1X Antibiotic–Antimycotic (15240062) HUVECs were purchased from Lonza (CC-2517) and cultured in VascuLife® VEGF Endothelial Medium (LifeLine, LL-0003).

Capsule Freezing and Recovery

After encapsulation, capsules were equilibrated at 37 °C in complete media overnight. Freezing process: 30-40 capsules were incubated at room temperature for 15 min in 500 μl of the intended CPA formulation and then frozen under gradient cooling conditions of 1 °C / min to − 80 °C. Recovery process: cryovials containing the frozen capsules were thawed quickly in a 37 °C water bath and the CPA formulation was completely removed. The capsules were then washed with 1 ml filtered phosphate buffer saline (PBS) and incubated at 37 °C in complete media for 24 h before characterization.

Free Cell Cryopreservation and Viability Measurement

RPE cells were frozen at a concentration of 1e6 cells/ml in preservation media under gradient cooling of 1 °C/min to − 80 °C. Recovery: cells were thawed in a 37 °C water bath and washed with PBS. Then, cells were spun down, resuspended in 1 ml complete media, and counted in 0.4% trypan blue stain for cell viability using an automated cell counter.

Live/Dead Staining of Cell Encapsulated Capsules

Capsules (n = 4–6) were incubated with 60 μl of the live dead stain made of 2 μM Calcein AM (Thermofisher Scientific, cat. no. C1430) and 4 μM EthD-1 (Thermofisher Scientific, cat. no. E1169) at 37 °C in the dark for 30 min. The stain was then discarded, and the capsules were washed with PBS before imaging.

Widefield Epifluorescence Imaging

Widefield Epifluorescent imaging of the capsules was performed on an inverted Nikon Eclipse Ti microscope (Nikon Instruments) equipped with a Phase Contrast Inverted Microscope 4x Objective (Plan Fluor 4x, NA 0.75) and controlled using NIS Elements AR software (Nikon Instruments). Emitted Calcein AM light (Thermofisher Scientific, cat. no. C1430) was imaged using an emission wavelength range of 468–552, and emitted EthD-1 (Thermofisher Scientific, cat. no. E1169) light was imaged using an emission wavelength range of 584-664 nm. Emitted light was imaged with an Andor Luca-R EMCCD Camera (Andor Technology). Images were acquired at 14-bit depth, 1002 × 1004 pixels, and pixel size 3.98 μm. For emitted Calcein-AM light, images were

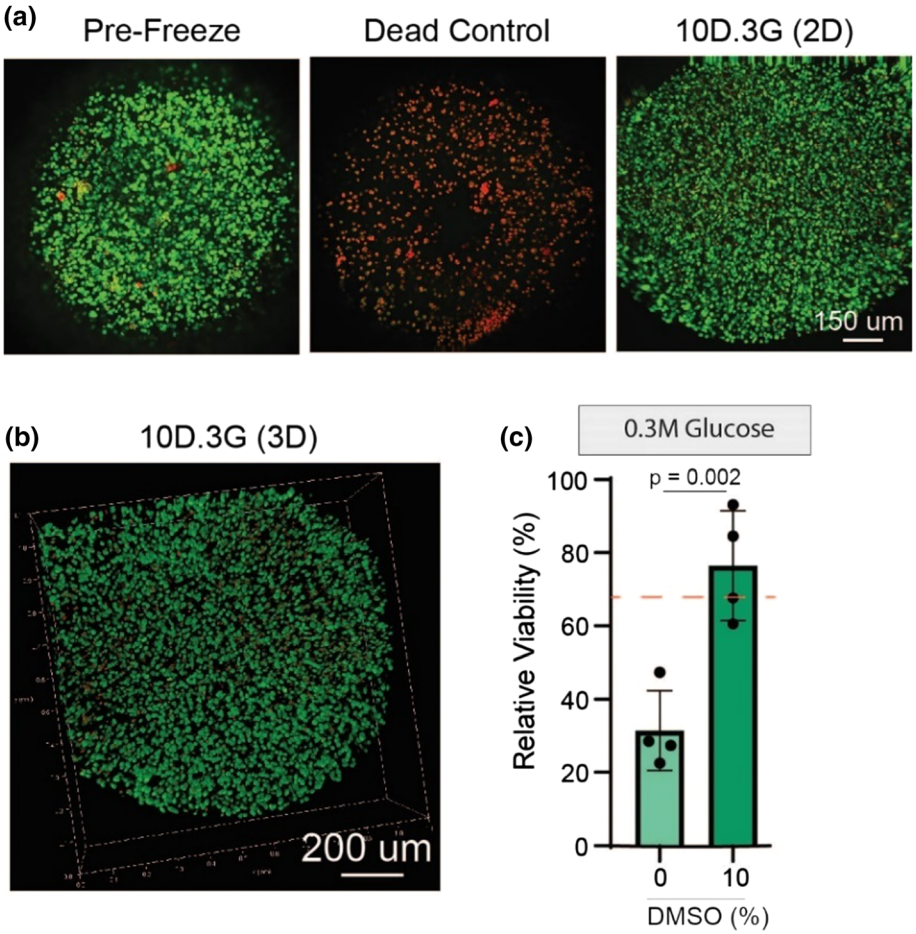


FIGURE 5. Spatial analyses of cell viability cryopreserved capsules. (a and b) Confocal images of pre-freeze, dead control, or 10D.3G capsules stained with calcein am (live) and ethidium homodimer-1 (dead). Z-Stacks were acquired on a Leica TCS SPE microscope at 10x magnification with 1024 × 1024 resolution and a step size of 1. Intensity for the GFP and Red channels were set to 20. Maximum projections were developed in FIJI, and overlayed in photoshop. 3D renderings were generated using the Leica software. 2D scale bars represent 150 μm. Each image is representative of 2–3 capsules imaged. 3D scale bar represents 200 μm. (c) Relative RPE viability 24 h post capsule thaw. Capsules were frozen in 10D.3G with or without 10% DMSO for 2 weeks. Each data point represents an individual capsule. Red dashed line represents the 70% viability threshold. P values were acquired using one way ANOVA with Holm-Sidak method for multiple comparisons.

captured using an exposure time of 1 s and for emitted Ethidium Homodimer-1 light, images were captured using an exposure time of 200 ms.

Image Processing

Raw dual-channel images were initially processed using NIS Elements Viewer (Nikon Instruments) in order to be separated into two independent TIFF files corresponding to Calcein-AM and Ethidium Homodimer-1 emitted light.

Using ImageJ, for each capsule and for each channel, a ROI was selected within the capsule that excluded any visible cells and was used to establish a representative area corresponding to "background" signal. The mean (μ) and standard deviation (σ) pixel intensities were determined for this ROI. As the pixel intensity of this ROI followed an apparently normal distribution, a cutoff pixel intensity of $\mu + 3\sigma$ was established for the entire image.

Next, pixel locations where the same pixel in both images was determined as coming from cells were determined using a Matlab script and subsequently relabeled as background in both images. This was

performed for both image channels resulting in two images per capsule where the 255-pixel values were now filtered to correspond to "true" Calcein-AM signal derived from cellular staining, and the other which would correspond to "true" Ethidium Homodimer-1 signal derived from cellular staining.

Confocal Imaging

Z-Stacks were acquired on a Leica TCS SPE microscope at 10x magnification with 1024 × 1024 resolution and a step size of 1. Intensity for the GFP and Red channels were set to 20. Maximum projections were developed in FIJI, and overlayed in photoshop. 3D renderings were generated using the Leica software.

Cell Viability Quantification from Live/Dead Staining

Utilizing the processed TIFF files, the number of 255-pixels for each channel was calculated using ImageJ. We refer to these quantities as Calcein Area and EthD1 Area. Next, we assumed that the Calcein-Am signal is proportional to the number of live cells and that the Ethidium Homodimer-1 signal is proportional to the number of dead cells. Thus, we calculated the encapsulated cell viability was determined as follows:

$$proportion\ of\ live\ cells = \frac{Calcein\ Area}{Calcein\ Area + EthD1\ Area}$$

Cell Viability Quantification Using Capsule Lysis

To manually quantify cell viability, 10 capsules from each sample were gently lysed using 200 μl alginate lyase for 30 min at 37 °C. The cells were recovered after the matrix was fully digested and then were counted using a 1:1 ratio of 0.4% trypan blue and an automated cell counter. This was done in duplicate for each sample during each experiment.

Cell Adherence Assay Following Capsule Lysis

After capsule lysis, the remaining cell suspension was added to 1 ml complete media and spun down at 250 g for 5 min. The supernatant was removed, and the pellet was resuspended in a fresh 1 ml of complete media and added to 1 ml complete media in a 6-well plate. The plates were left in a 37 °C incubator for 24 h to allow the cells to adhere. The supernatant was then collected, and the floating/dead cells were counted using the trypan blue method described above. 1 ml PBS was added to each well and the plates were imaged at 20x using dark field microscopy to visualize cell adherence following cryopreservation, thawing, and lysis.

Therapeutic Protein Production Measurement

Post recovery, 200 μl complete media was added to each capsule from different preservation media and incubated at 37 °C for 24 h. The supernatants were collected and stored at − 20 °C. ELISA of mouse IL-12 (R&D Systems, cat. M1270) and mouse IL-2 (R&D Systems, cat. M2000) were performed to measure the therapeutic protein production from the capsules.

Statistics

Experiments were repeated at least once, or data were compiled from two independent experiments unless otherwise stated in the respective figure legend. Replicates were reproducible. All statistical analyses were conducted using GraphPad Prism 9. One-way ANOVA tests with the Holm-Sidak multiple comparisons methods were used to determine p values for appropriate datasets.

CITATION DIVERSITY STATEMENT

Recent work has identified bias in citations, where minority groups have been under-cited relative to the number of publications in the field. We recognize this bias and have worked diligently to ensure that we reference appropriate papers with fair gender and racial author inclusion.

SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at https://doi.org/10.1007/s12195-022-00739-7.

ACKNOWLEDGMENTS

This study was funded by an award from the National Institute of Health (1RO1DK120459), and the National Science Foundation (CBET2041107), as well as by a National Science Foundation fellowship (1842494) to A. Nash.

CONFLICT OF INTEREST

Yufei Cui, Bertha Castillo, Leonardo D. Sanchez Solis, Samira Aghlara-Fotovat, Maya Levitan, Boram Kim, and Michael Diehl declare that they have no

conflict of interest. Amanda M. Nash declares stock ownership in Avenge Bio. Omid Veiseh declares stock ownership and consulting income from Avenge Bio, Sigilon Therapeutics, and Pana Bio.

ETHICAL APPROVAL

This study did not involve any human subjects or animal research

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