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Slow-delivery and distributed exchange of cryoprotective agents with hydrogel beads

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ABSTRACT

Intracellular loading of cryoprotective agents (CPAs) into target cells is a critical step for cryopreservation. However, biological membranes are usually much less permeable to CPAs than to water, resulting in high osmotic pressures and osmotic damage during the CPA loading and unloading phases of cryopreservation. Here, we show that calcium alginate hydrogel beads several millimeters in diamater containing CPAs can be admixed with a cell suspension to spontaneously release CPAs in a gradual and distributed manner. We demonstrate that beads containing cell media enable the gradual removal of CPA from Jurkat cells equilibrated in a typical cryopreservation solution of 15% glycerol, protecting the cells from hypotonic damage. We show that the dynamics of CPA exchange are accurately described by a numerical model of free diffusion within the gel. This approach may enable semiautomated and closed methods of gradual CPA exchange from large volume cell suspensions.

Cell-penetrating cryoprotective agents (CPAs) are required to prevent the formation of intracellular ice during cryopreservation by slow cooling of mammalian cell suspensions [7]. Common cryopreservation medium formulations use high volume fractions (e.g. >10%) of miscible solvents such as dimethyl sulfoxide (Me_2SO), propanediol, or glycerol that diffuse across lipid bilayer membranes. However, the plasma membranes of most cells are much less permeable to these CPAs than they are to water, especially in the presence of water-specific aquaporins [6,8]. Care must therefore be taken to prevent excessive hypertonic and hypotonic shocks when washing cell suspensions into and out of such cryo media, respectively. Unfortunately, current clinical methods for sterile processing of large sample volumes do not prevent this mechanism of cytotoxicity during the CPA loading and unloading phases of cryopreservation.

In the laboratory, gradual dropwise addition of concentrated CPAs is commonly used to prevent hypertonic shock during CPA loading. Likewise, gradual dropwise dilution with isotonic medium is standard practice for washing thawed cells. Unfortunately, these laboratory practices are cumbersome and not so easily scaled to larger sample volumes, such as those used for various clinical applications in cell therapy, which are commonly in the range of 10–100 mL containing hundreds of millions to billions of cells [4]. As the sample volume increases, it becomes more challenging to rapidly and uniformly mix CPAs into the solution without exposing cells to large shear stresses, especially when concentrated CPAs have a different density and viscosity than the medium. Additionally, clinical best practice for many cell therapies is to directly transfuse the thawed cell suspension without washing, due to the unreliable recovery rate of centrifugal cell washing before transfusion (e.g. 60% cell recovery for hematopoietic stem cells) [1]. Regrettably, this medically unnecessary exposure to large amounts of Me_2SO results in high rates of adverse events [2,3]. Therefore, the growing clinical use of precious patient-derived and/or engineered therapeutic cells underscores the need for methods and materials to mediate the gradual exchange of small molecule CPAs from large-volume cell suspensions to minimize cellular damage [5]. Furthermore, these therapeutic cells need to be processed at the point of care and not at an advanced medical center, which could greatly benefit from a robust, automated single-step protocol.

Here, we investigated the use of calcium alginate hydrogel beads to gradually and homogeneously release and/or absorb CPAs from the cell suspension. In this approach, millimeter-scale hydrogel beads are impregnated with concentrated CPA(s), and then gently mixed with the cell suspension. CPAs were found to readily diffuse from within the gel into the surrounding medium and are equilibrated over the course of several minutes. The characteristic rate of CPA release increases for larger beads, as this increases the characteristic length scale of diffusion

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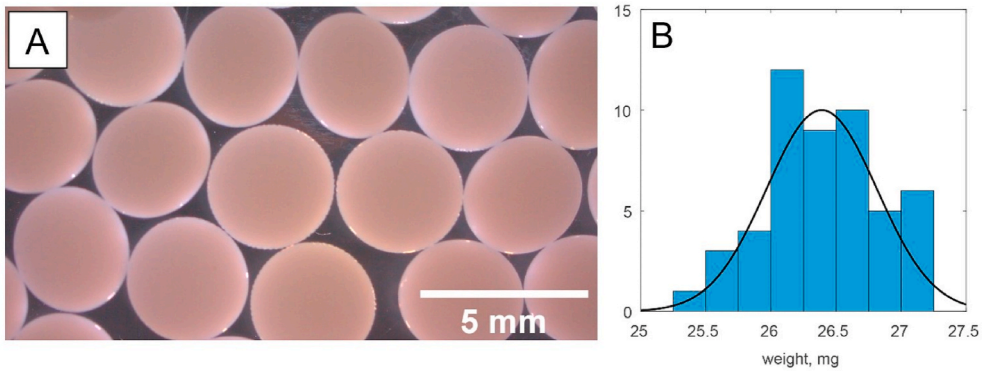

Fig. 1. (A) Calcium alginate hydrogel beads for controlled CPA uptake and release. (B) Distribution of bead weights (N = 50) with fitted Gaussian. The average bead diameter is about 3.7 mm.

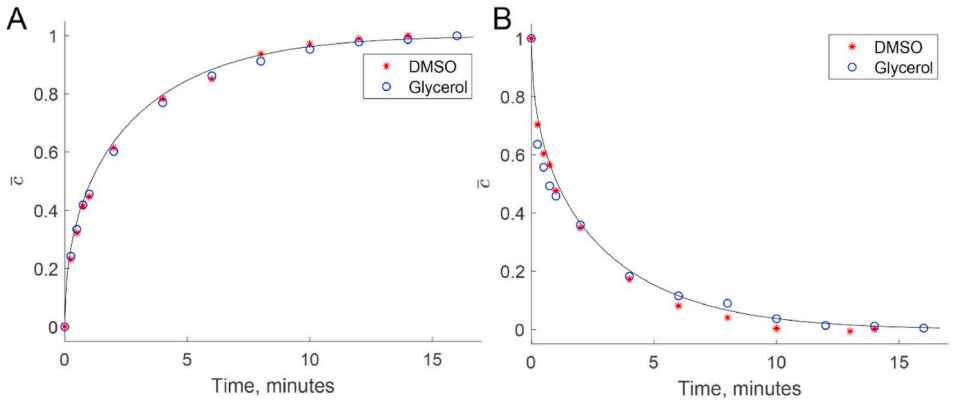

Fig. 2. Rates of cryoprotective agent (A) uptake and (B) release from calcium alginate beads. Solid line is the solution to numerical model, parameters in text.

within the bead. For spherical hydrogel beads, the characteristic rate is proportional to the square of bead diameter (Supporting Information). Controlling the average bead size, therefore, allows control over the average rate of CPA uptake and/or release.

To test whether hydrogel beads could gradually exchange common cell-penetrating CPAs with the surrounding solution, calcium alginate beads about 25 μL in volume were synthesized by the standard dripping approach [9]. 5% w/v alginic acid was dissolved in distilled water and dripped at a flow rate of 1 mL/min from a 15G needle into a bath of 200 mM $CaCl_2$. Beads were washed in 200 mM $CaCl_2$, then stored refrigerated in a solution of 200 mM $CaCl_2$ for up to 1 month. The beads were measured to be about 3.6 mm in diameter, with an average mass of about 26.4 mg and a coefficient of variation of about 2% (Fig. 1). Based on an expected density of 1.025 mg/cm^3, a mass of 26.4 mg corresponds to a sphere with diameter of 3.67 mm.

The rate of uptake and release of Me_2SO and glycerol from the beads was quantified by osmometry. To measure CPA release kinetics, approximately 3 g of beads were equilibrated with a 10% volume ratio solution of glycerol or Me_2SO in aqueous 50 mM $CaCl_2$ for at least 1 h. Beads were then removed with a spoon and/or by pouring the solution through a 50 μm plastic mesh cell strainer. Then, the outside of the cell strainer basket was blotted against paper tissue to absorb remaining excess liquid from the bead surface, and the beads spooned into a 5 mL bath of CPA-free 50 mM $CaCl_2$ solution in a standard 15 mL conical tube. The mixture was rocked gently on a motorized sample mixer for up to 16 min, and 50 μL samples (of the liquid fraction, with no beads) were removed at regular intervals. The osmolality of the samples was measured by micro-osmometer (Advanced Instruments 3300) to infer CPA concentration based on sample standards. The instrument coefficient of variation of the micro-osmometer was estimated to be less than 1%, based on replicated measurements of standard samples. The rates of CPA uptake into the hydrogel beads was determined identically, by mixing CPA-free beads with a solution of 10% v/v Me_2SO or glycerol and measuring solution osmolality at several timepoints. The normalized change in concentration over time of both uptake and release of glycerol and Me_2SO is shown in Fig. 2: $\bar{c}(t) = \frac{c(t)-c_0}{c_\infty - c_0}$ where c_0 and c_∞ are concentrations at $t = 0$ and $t = \infty$ respectively. The numerical solution of diffusion from a spherical bead is also shown, for a coefficient of diffusion within the hydrogel of 1.5e-5 cm^2/s and a bead volume 25 μL (Supporting Information). The values of bead size and the Coefficient of Diffusion used in the model were fitted parameters, and fall close to the experimental bead volume of 25.7 μL and the self-diffusion coefficient of water (approximately 2e-5 cm^2/s at room temperature). Altogether, the system was found to reach 50% of concentration equilibration after about 1 min, and 90% after about 6.5 min.

To test whether gradual exchange of CPA using hydrogel beads could prevent osmotic damage, we developed a room temperature model of CPA-induced hypotonic shock in Jurkat cells, a CD4 T cell line. Jurkat cells were equilibrated with a cryopreservation solution of 15% v/v glycerol in tris-buffered saline with 10 mM $CaCl_2$ ('TBS-C') by gentle rocking for 10 min at room temperature in 15 mL conical tubes, at a concentration of about 750,000 cells in 1.5 mL. The additional calcium ion was included to prevent any changes to calcium alginate gel cross-linking. After 10 min incubation, cells were then diluted in room temperature glycerol-free TBS-C either by single-step 10x dilution, or in three sequential dilutions of 2.15x at 5 min intervals (i.e., sequential additions of 1.75 mL, 3.75 mL, and 8 mL, resulting in a final volume of 15 mL and final dilution factor of 10x). Cells were then washed by centrifugation into complete cell culture medium (RPMI 1640 with 10% fetal bovine serum) and incubated at 37 °C. After 4 h, cell number and

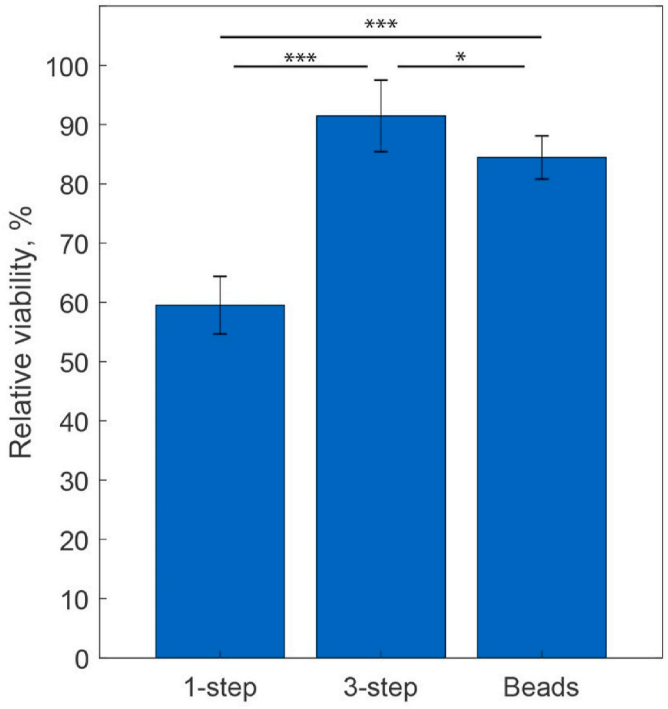

Fig. 3. Relative viability of Jurkat cells loaded with 15% glycerol (N = 5) and then unloaded by conventional dilution (1-step, 3-step), or with media exchange using hydrogel beads. ***: p < 1e-5, *: p = 0.03.

viability were determined by automated cell counting of propidium iodide exclusion: cells were centrifuged and resuspended in 1e6/mL of PBS +2 μg/mL propodium iodide and incubated for at least 5 min before automated cell counting and viability analysis (Nexcelom Cellometer).

To test whether glycerol removal by hydrogel beads would prevent cell death from CPA-induced osmotic shock, the above procotol was modified to use glycerol-free hydrogel beads instead of direct dilution to remove glycerol from the cells. Beads as described earlier were equilibrated with glycerol-free TBS-C buffer for at least 30 min, and then blotted to remove excess surface liquid and weighed. An equal mass of beads were mixed gently with the cell suspension (e.g. 1.5 mL of cells in glycerol +1.5 g of glycerol-free beads). After 5 min, the cell suspension was collected by pipette from around the beads, and transferred to a second equal mass of beads prepared identically, and gently mixed for an additional 5 min, resulting in a total dilution factor of 4x. Viability for each condition was normalized to a control aliquot of cells kept at room temperature but otherwise untreated during the experiment, which was within 94%–96% for all replications. Single replicates of all experimental conditions (control, single-step dilution, 3-step dilution, and bead-mediated dilution) were run sequentially on the same day. Replicates were obtained by performing the identical protocol on five separate days.

Both the 3-step dilution and the hydrogel bead mediated dilution resulted in significantly higher viability compared to the 1-step dilution, as measured by single-sided *t*-test (Fig. 3). The bead dilution condition was also significantly different than the 3-step dilution, as measured by a two-sided *t*-test. No corrections were made for multiple comparisons.

This work represents a slow delivery approach using diffusion-limited transport to load and unload CPAs to cells in and out of hydrogel beads, respectively. This approach can prevent cell death induced by hypotonic shock, similarly to laboratory-standard dropwise unloading but with reduced user intervention. A distinguishing feature of this approach is that it allows controlled-rate exchange of CPAs from large sample volumes without exposing cells to significant shear forces. In the future, we envision that this approach may be integrated into a closed system for semi-automated CPA exchange.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cryobiol.2021.09.006.

References

[1] B. Calmels, P. Houzé, J.-C. Hengesse, T. Ducrot, C. Malenfant, C. Chabannon, Preclinical evaluation of an automated closed fluid management device: cytomate TM, for washing out DMSO from hematopoietic stem cell grafts after thawing, Bone Marrow Transplant. 31 (9) (May 2003) 823, https://doi.org/10.1038/sj.bmt.1703905.

[2] M.A. Cox, J. Kastrup, M. Hrubiško, Historical perspectives and the future of adverse reactions associated with haemopoietic stem cells cryopreserved with dimethyl sulfoxide, Cell Tissue Bank. 13 (2) (Jun. 2012) 203–215, https://doi.org/10.1007/s10561-011-9248-2.

[3] C.R. Cruz, et al., Adverse events following infusion of T cells for adoptive immunotherapy: a 10-year experience, Cytotherapy 12 (6) (Oct. 2010) 743–749, https://doi.org/10.3109/14653241003709686.

[4] N.J. Dwarshuis, K. Parratt, A. Santiago-Miranda, K. Roy, Cells as advanced therapeutics: state-of-the-art, challenges, and opportunities in large scale biomanufacturing of high-quality cells for adoptive immunotherapies, Adv. Drug Deliv. Rev. 114 (May 2017) 222–239, https://doi.org/10.1016/j.addr.2017.06.005.

[5] M.A. Fischbach, J.A. Bluestone, W.A. Lim, Cell-based therapeutics: the next pillar of medicine, Sci. Transl. Med. 5 (179) (Apr. 2013), https://doi.org/10.1126/scitranslmed.3005568, 179ps7-179ps7.

[6] F.W. Kleinhans, "Membrane permeability modeling: kedem–katchalsky vs a two-parameter formalism, Cryobiology 37 (4) (Dec. 1998) 271–289, https://doi.org/10.1006/cryo.1998.2135.

[7] D.E. Pegg, Principles of cryopreservation, in: J.G. Day, G.N. Stacey (Eds.), Cryopreservation And Freeze-Drying Protocols, Humana Press, Totowa, NJ, 2007, pp. 39–57, https://doi.org/10.1007/978-1-59745-362-2_3.

[8] L. Weng, et al., A highly-occupied, single-cell trapping microarray for determination of cell membrane permeability, Lab Chip 17 (23) (2017) 4077–4088, https://doi.org/10.1039/C7LC00883J.

[9] L. Weng, S.N. Tessier, A. Swei, S.L. Stott, M. Toner, Controlled ice nucleation using freeze-dried Pseudomonas syringae encapsulated in alginate beads, Cryobiology 75 (Apr. 2017) 1–6, https://doi.org/10.1016/j.cryobiol.2017.03.006.