

# Low DMSO Cryopreservation of Stem Cells Enabled by Macromolecular Cryoprotectants

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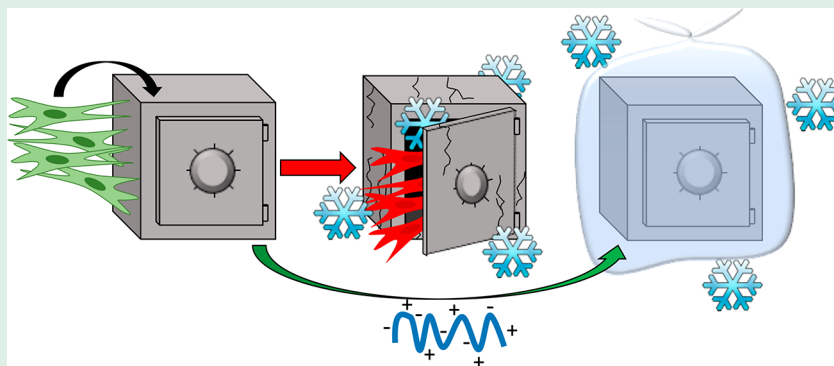
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**ABSTRACT:** Mesenchymal stromal (stem) cells have potential in regenerative medicine and modulating the immune system. To deliver any cell-based therapy to the patient, it must be cryopreserved, most commonly in DMSO, which impacts cell function and causes clinical side effects. Here we report the use of a synthetically scalable polyampholyte to rescue the cryopreservation of mesenchymal stromal cells in low [DMSO] cryopreservation. Flow cytometry showed retention of key markers of multipotency comparable to 10% (v/v) DMSO, and the cells could be differentiated, showing this polymer material can be used to improve, or replace, current cryopreservation strategies.

**KEYWORDS:** cryopreservation, stem cells, biomaterials, polymers, polyampholyte

Mesenchymal stromal (stem) cells (MSC) have huge potential as regenerative therapies for a range of indications from treating fibrosis, rescuing heart function, and modulating the immune system.<sup>1</sup> A key aspect in the development of MSC treatments is producing and storing large quantities of cells, since they have a limited lifetime in vitro and cannot be continuously cultured due to phenotype drift.<sup>2</sup> Isolated cells must be transported to special processing facilities, expanded and, crucially, cryopreserved for storage and transport to clinics. To enable future clinical therapies to succeed, it is imperative that cell characteristics are recovered after cryopreservation. Current methods to store MSCs (and most cells) rely on high volumes (10% v/v) of dimethyl sulfoxide (DMSO), which has been shown to cause phenotypic changes in stem cells<sup>3</sup> and can induce differentiation in embryonic stem cells at concentrations as low as 0.125%.<sup>4</sup> More critically, DMSO is known to cause adverse side effects in patients receiving cell therapies, ranging from mild symptoms, such as nausea,<sup>5</sup> to seizures and cardiac arrest.<sup>6</sup> A study reported one in 70 transfusions result in DMSO-related side effects.<sup>7</sup>

As a result, there is interest in the development of cryoprotective agents that can reduce the amount of DMSO in the cryoprotective solution while recovering large numbers

of viable cells and retaining cell-specific functions. In the case of stem cells, this means preserving stem cell characteristics such as specific surface markers and differentiation capacity. Alternative cryopreservation methods include using other small molecules such as glycerol and trehalose,<sup>8</sup> or through vitrification whereby large volumes of solvent are used to achieve a glassy state without ice formation. However, vitreous states can be unstable, leading to ice nucleation followed by catastrophic ice growth, and the high concentrations of solvents required can lead to toxicity.<sup>9</sup>

As an alternative to the traditional small-molecule-based cryoprotectants, macromolecular (polymer/protein) cryoprotectants are emerging,<sup>10,11</sup> inspired by ice binding,<sup>12</sup> ice nucleating,<sup>13</sup> and late embryogenesis abundant proteins<sup>14</sup> found in nature. The function of macromolecular cryoprotectants includes ice recrystallization inhibition (IRI) activity<sup>15</sup>

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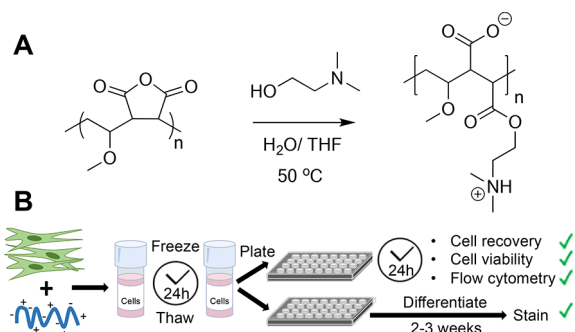
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or potentially osmotic regulation.<sup>17</sup> One emerging class of macromolecular cryoprotectants is polyampholytes, which contain both cationic and anionic side chains, which have been shown to be potent cryoprotectants. Their mechanism of action remains unclear, but it has been suggested to involve membrane stabilization.<sup>16</sup> Polyampholytes are reported to exhibit some IRI activity,<sup>18</sup> but it is weak when compared to potent IRI active polymers, such as PVA.<sup>19</sup> Matsumura et al. have used polyampholytes to vitrify induced pluripotent stem cells (iPSC) by using 10% (w/v) polymer along with 6.5 M ethylene glycol and 0.75 M sucrose.<sup>20</sup> Recoveries of >60% could be achieved when cells were incubated for 30 s with the vitrification solution, but this was more than halved when incubation times were extended to 120 s, due to the toxicity of such high volumes of solvent. In contrast, cell cryopreservation has been achieved with polyampholytes using slow-cooling methods, which are desirable due to low solvent concentrations, sample stability, and straightforward processes. We recently reported a polyampholyte cryoprotectant that was a potent additive for the cryopreservation of cell monolayers (demonstrated in multiple immortalized cell lines) using slow-cooling, which is an extremely challenging model.<sup>21</sup>

Here, we study the scope and limitations of this synthetically scalable polyampholyte to cryopreserve mesenchymal stromal cells in suspension. It is shown that the polymer allows successful cryopreservation at just 2.5% (v/v) DMSO and that the recovered cells are viable, retain all “stem-like” markers, and are capable of differentiation into various lineages.

The polyampholyte (cryoprotectant) was synthesized by the reaction of poly(methyl vinyl ether-*alt*-maleic anhydride) with dimethylamino ethanol (Figure 1A). Both components are low



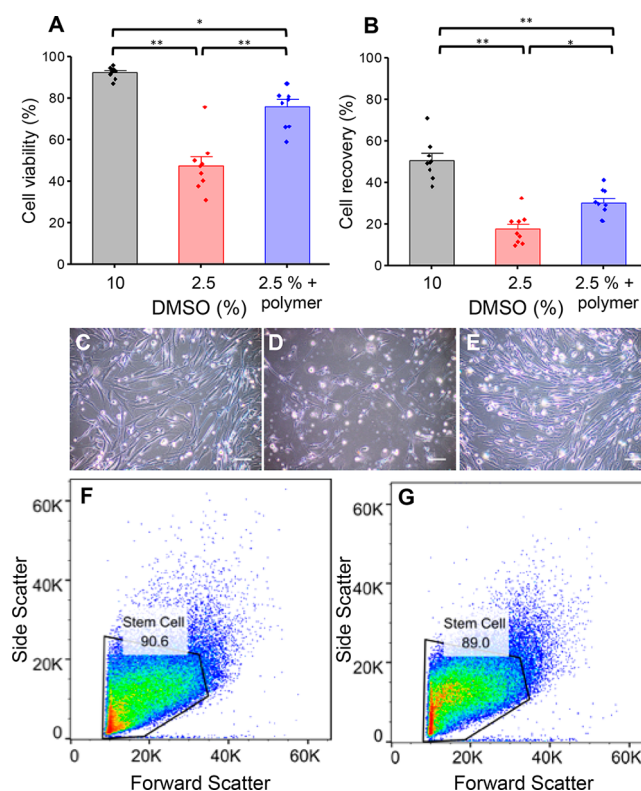
**Figure 1.** (A) Synthesis of polyampholyte. (B) Cryopreservation protocol for hBM-MSC slow freezing method and analysis.

cost and available to GMP (good manufacturing practice) grade, on a scale necessary for a new cryoprotectant to be useful. This point is essential as just one liter of cells would require ~100 g of a cryoprotectant at 10% (w/v) and hard-to-synthesize or peptide-based materials are typically not available at this scale. The use of a maleic anhydride precursor polymer also guarantees an alternating monomer sequence due to its propensity to cross-propagate and a 1:1 cationic/anionic ratio (crucial for cryopreservation success), which cannot be achieved by random copolymerization.<sup>22</sup> This polymer is a potent cryoprotectant for cell monolayers;<sup>21</sup> its mode of action does not involve vitrification, and it has only weak ice recrystallization inhibition activity, with evidence that it can stabilize cell membranes.<sup>16,21</sup>

Human bone marrow-derived mesenchymal stromal (stem) cells (hBM-MSC) were initially screened for cryopreservation

conditions (Figure S1). Cells were cooled at 1 °C min<sup>-1</sup> to -80 °C, transferred to liquid nitrogen for 24 h then thawed at 37 °C in a water bath, plated, and allowed to recover for 24 h before analysis. Initial cryopreservation screens used 1 × 10<sup>5</sup> cells mL<sup>-1</sup> and variable concentrations of DMSO/polyampholyte. This analysis revealed that 20 mg mL<sup>-1</sup> of the polyampholyte was needed to enable a reduction in the DMSO to 2.5% (v/v) but that the total number of cells recovered post-thaw was rather low (<10% recovered). From this point, an increased cell density of 5 × 10<sup>5</sup> cells mL<sup>-1</sup> was used, as this gave close to 50% recovery in standard (10% (v/v) DMSO) conditions.

Figure 2 shows the results of the optimized hBM-MSC cryopreservation. When the concentration of DMSO was



**Figure 2.** hBM-MSC post-thaw recovery after 24 h. (A) Cell viability. (B) Total number of cells recovered, polymer indicates 20 mg mL<sup>-1</sup> polyampholyte. Viability and recovery data are expressed as mean ± SEM for three independent experiments. Statistical analysis by one-way ANOVA, \* =  $p < 0.05$ , \*\* =  $p < 0.0001$ . Light microscope images of cells 24 h post-thaw when frozen with (C) 10% (v/v) DMSO, (D) 2.5% (v/v) DMSO, (E) 2.5% DMSO (v/v) + 20 mg mL<sup>-1</sup> polyampholyte. Scale bar indicates 100 μm. Flow cytometry data showing forward and side scatter of thawed hBM-MSCs; (F) 10% DMSO (v/v), (G) 2.5% DMSO (v/v) + 20 mg mL<sup>-1</sup> polyampholyte.

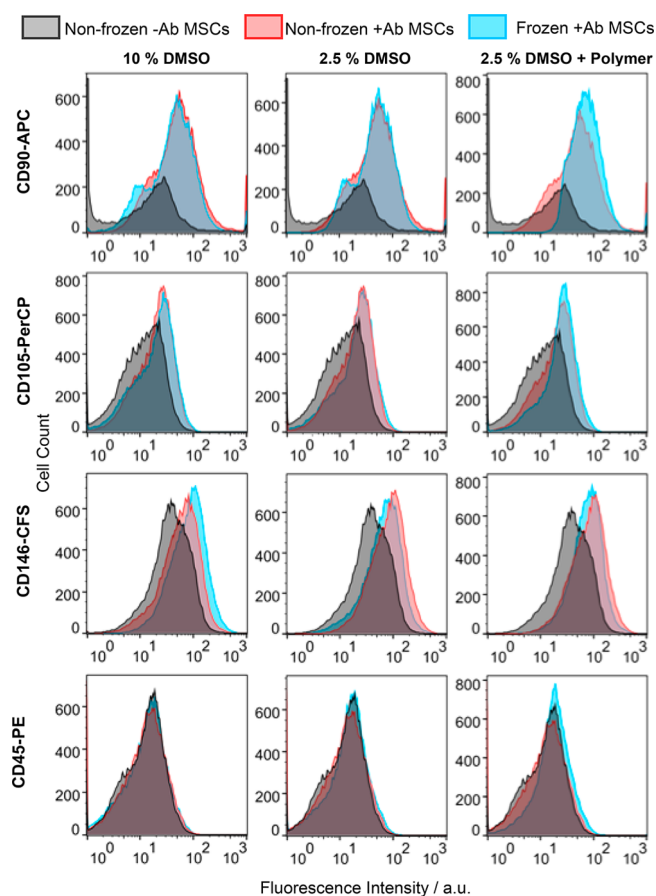
reduced from 10 to 2.5% (v/v), the post-thaw viability of hBM-MSC dropped from 92 to 47% (Figure 2A), highlighting why such high concentrations of DMSO are essential to protect the cells. However, upon supplementing with 20 mg mL<sup>-1</sup> polyampholyte, the viability was rescued to 76%, demonstrating that the polyampholyte is a potent cryoprotectant. Antebi et al. reported the 24 h post-thaw viability of MSC frozen with 10% DMSO to be ~80%, highlighting that supplementation with our polyampholyte can achieve the same results but using just a quarter of the [DMSO].<sup>23</sup> In a clinical

context, this is a highly desirable achievement, as lowering the DMSO content of transfused stem cells has been shown to reduce the incidence of adverse clinical side effects 5-fold.<sup>7</sup> Critically, we have reported 24 h post-thaw results as MSCs require 24 h post-thaw to regain function and enable true evaluation of recovery.<sup>23</sup> This point is crucial to show the impact of this work; Zhao et al. reported post-thaw viabilities of ~90% when freezing 3T3 cells with polyampholytes. However, the majority of cells died after 24 h in culture.<sup>17</sup> Similarly, Naaldijk et al. reported immediate post-thaw viabilities of MSC frozen with DMSO and hydroxyethyl starch to be ~85%, but this plummeted to <30% after 3 days in culture for samples containing <4% DMSO.<sup>24</sup> These studies highlight that immediate post-thaw viability is not a good predictor of cell health.<sup>25</sup>

While viability is the most widely reported measure of cryopreservation, it does not consider the fraction of cells lost due to lysis and mechanical damage. Therefore, the post-thaw total cell recovery values (which compare the number of cells initially frozen to the number of live cells present after thawing) were measured (Figure 2B). When frozen with 10% (v/v) DMSO, 51% of hBM-MSCs were recovered 24 h post-thaw. This dropped to just 17% when 2.5% (v/v) DMSO was used as the cryoprotectant. The addition of 20 mg mL<sup>-1</sup> polyampholyte doubled the post-thaw recovery to 30%, clearly seen with microscopy of cells 24 h post-thaw (Figure 2C–E). Very few other studies count recovered cells. However, Petrenko et al. reported that 5% DMSO led to cell recoveries of <25% in hematopoietic stem cells,<sup>26</sup> and Verdanova et al. showed that 5% DMSO gave 36% recovery of human MSCs.<sup>27</sup> This clearly highlights that our polyampholyte enables a significant improvement in both the viability and recovery of hBM-MSC at low concentrations of DMSO.

Flow cytometry of thawed cells revealed minimal differences in both size (forward scatter) and granularity (side scatter) when comparing cells subjected to freezing in 10% versus 2.5% (v/v) DMSO supplemented with 20 mg mL<sup>-1</sup> polyampholyte (Figure 2F,G). Under all conditions, approximately 90% of all recovered cells were located within the gated viable cell region. Thus, supplementation of this polyampholyte to cryopreservation solutions clearly leads to post-thaw recovery of intact cells, using significantly reduced [DMSO].

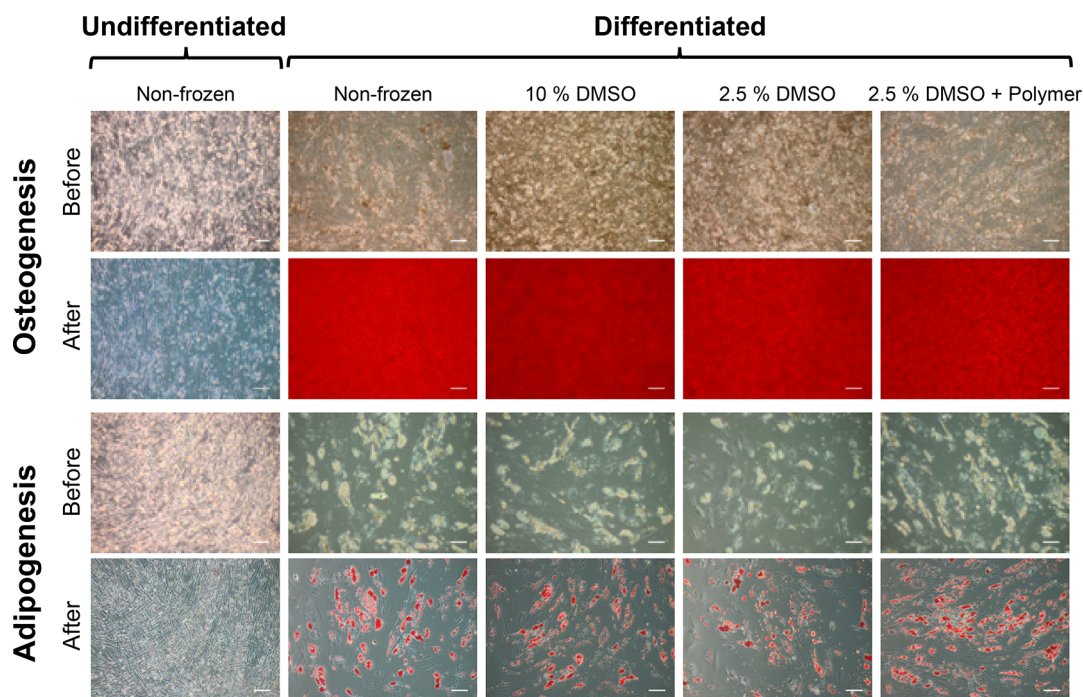
The above showed that large numbers of viable cells are recovered when the polyampholyte was included, but it is crucial to demonstrate MSC function, especially for biomedical applications. Therefore, thawed hBM-MSCs were assessed for the presence of stem cell surface markers that indicate multipotency: CD90, CD105, and CD146, as well as the absence of CD45 (negative marker, not present on MSCs).<sup>28</sup> Surface marker expression was studied using antibodies conjugated to fluorescent dyes and analyzed by flow cytometry. All samples were compared to non-frozen controls (Figure 3) and isotype controls (Figure S3). In comparison to non-frozen antibody untreated (–Ab) hBM-MSC controls, a clear enhancement in the fluorescence intensity of positive stem cell surface markers was observed in non-frozen antibody-treated (+Ab) hBM-MSC controls and hBM-MSCs frozen in 10% (v/v) DMSO, 2.5% (v/v) DMSO, and 2.5% (v/v) DMSO plus polyampholyte. However, no change was seen when the same cells were treated with anti-CD45 antibodies, demonstrating the absence of this negative marker. Crucially, no significant changes to the mean fluorescence intensity of cells frozen in the different cryoprotectants were observed



**Figure 3.** Flow cytometry analysis of immunostained hBM-MSCs frozen in the presence of 10% (v/v) DMSO, 2.5% (v/v) DMSO, and 2.5% DMSO (v/v) + 20 mg mL<sup>-1</sup> polyampholyte. Recovered cells were immunostained for cell surface markers using 3 positive markers (CD90-APC, CD105-PerCP, and CD146-CFS) and 1 negative marker (CD45-PE). Nonfrozen hBM-MSCs, both unstained (–Ab) and stained (+Ab), were provided as controls to observe changes in fluorescence intensity.

compared to nonfrozen + Ab hBM-MSC controls, Figure S4, indicating that freezing in DMSO and polyampholyte does not significantly change the expression of stem cell surface markers and hence the multipotent identity of hBM-MSCs.

As a final test, differentiation experiments were undertaken to understand if the cells retained their multilineage capacity. Immediately after thawing, cells were added to gelatin-coated plates and incubated for 24 h before treatment with either osteogenic differentiation media or adipogenic differentiation media for 2–3 weeks. Following incubation, cells were stained with Alizarin Red S for the presence of calcium deposits produced by osteoblasts and Oil Red O to identify lipid droplets present in adipocytes, Figure 4. All samples treated with osteogenic differentiation media showed clear differentiation into an osteoblast (bone) cell phenotype as demonstrated by extensive calcium deposits, stained bright red by Alizarin Red S staining, Figure 4. Negligible differences could be identified between samples cryopreserved in each of the different cryopreservation solutions as well as the nonfrozen control. Calcium deposits were absent in undifferentiated control cells, showing there was no premature/spontaneous differentiation. Similarly, all samples treated with adipogenic differentiation media displayed an adipocyte (fat) cell phenotype, indicated by the presence of lipid droplets that



**Figure 4.** hBM-MSC differentiated toward osteoblast (osteogenesis) and adipocyte (adipogenesis) phenotypes and stained with Alizarin Red S and Oil Red O, respectively. Scale bar, 100  $\mu\text{m}$ . Images show the same sample before and after staining. Undifferentiated and differentiated nonfrozen controls were included for comparison. Remaining samples were cryopreserved with 10% (v/v) DMSO, 2.5% (v/v) DMSO, or 2.5% (v/v) DMSO + 20  $\text{mg mL}^{-1}$  polyampholyte.

were stained positively with Oil Red O, as well as a flattened cell morphology. Again, no difference was found between nonfrozen samples and samples cryopreserved with the three cryoprotectants. Oil Red O staining was absent for undifferentiated control cells. Figure 4 clearly demonstrates that hBM-MSC cryopreserved in the presence of the polyampholyte is capable of differentiating into both osteoblast (bone) and adipocyte (fat) cells, giving the same results as cells cryopreserved in 10% (v/v) DMSO but with a quarter of the permeable cryoprotectant.

In summary, we have demonstrated that a polyampholyte, which is obtained on a large scale from a commodity polymer precursor, allows the use of significantly lower DMSO concentrations for human mesenchymal stromal (stem) cell (hBM-MSC) cryopreservation. Just 20  $\text{mg mL}^{-1}$  of the polyampholyte enabled the rescue of MSC cryopreservation when the DMSO was lowered from the standard 10% (v/v) to just 2.5% (v/v). Flow cytometry analysis confirmed that the stromal/stem cell surface markers CD90, CD105, and CD146 were all retained and were statistically identical between cells stored in 10% (v/v) DMSO, compared to 2.5% (v/v) DMSO + polyampholyte. Furthermore, the thawed cells were successfully differentiated into osteogenic and adipocyte lineages, confirming their “stemness”. These results show that significant reductions in DMSO concentration can be achieved in biomedically relevant cell lines by using polyampholytes and, importantly, do not require large solvent volumes associated with vitrification, which has been reported for other macromolecular cryoprotectants. We also show an improvement in the total number of cells being recovered, compared to the standard method where only the viability is reported, which can overestimate the cryopreservation outcome. These results will aid the development of advanced

cryoprotective formulations for cell-based therapies as well as basic biomedical science.

## EXPERIMENTAL SECTION

Full details of experimental procedures are given in the [Supporting Information](#). Polyampholyte was synthesized as previously described.<sup>21</sup>

**Cryopreservation of Cell Suspensions.** The polymer was dissolved in culture media containing 60% FBS and 5% DMSO, at 2 $\times$  the final concentration, and then sterile-filtered through a 0.2  $\mu\text{m}$  membrane. Cells were treated with Accutase for 10 min at room temperature, neutralized with complete cell media, and centrifuged for 5 min at 180g. Following centrifugation, cells were diluted 1:1 with 0.4% trypan blue and counted with a hemocytometer to obtain the number of viable cells. The cell density was adjusted to  $1 \times 10^6$  cells  $\text{mL}^{-1}$ , and a second cell count was performed to obtain an accurate prefreeze value. Cells were diluted 1:1 in cryoprotectant in cryovials (total volume 1 mL) and mixed 3 times. Triplicate samples were prepared for each cryopreservation solution: 10% DMSO, 2.5% DMSO, or 2.5% DMSO + 20  $\text{mg mL}^{-1}$  polyampholyte, all containing 30% FBS. The vials were placed in a CoolCell freezing box in a  $-80$   $^{\circ}\text{C}$  freezer for 2 h, with a freezing rate of  $1$   $^{\circ}\text{C min}^{-1}$ . After 2 h, the vials were transferred to a liquid nitrogen dewar for a minimum of 24 h. To thaw, vials were placed in a  $37$   $^{\circ}\text{C}$  water bath until nearly thawed; then, the contents were diluted 1:10 in complete cell culture media and centrifuged at 180g for 5 min. Cells were resuspended in 500  $\mu\text{L}$  complete cell media and then transferred to individual wells of a 0.1% gelatin-treated 24-well plate. Plates were incubated at  $37$   $^{\circ}\text{C}$  and 5%  $\text{CO}_2$  for 24 h. After 24 h, total cell recovery and cell viability were assessed by the trypan blue exclusion assay.

**Trypan Blue Exclusion Assay.** Cells were treated with Accutase for 10 min at room temperature and then centrifuged at 180g for 5 min. The cell pellet was resuspended in 500  $\mu\text{L}$  complete media; then, a sample was diluted 1:1 in 0.4% trypan blue and counted using a hemocytometer. Cell viability and cell recovery were determined using the two equations, where cell viability is the number of unstained cells post-thaw compared to the total cells post-thaw and

where cell recovery is the number of unstained cells post-thaw compared to the total cells initially frozen.

$$\text{viability}_{\text{TB}} (\%) = \frac{\text{cells}_{\text{unstained}}}{\text{cells}_{\text{unstained}} + \text{cells}_{\text{stained}}} \times 100$$

$$\text{recovery}_{\text{TB}} (\%) = \frac{\text{cells}_{\text{unstained}}}{\text{cells}_{\text{frozen}}} \times 100$$

## ■ ASSOCIATED CONTENT

### SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsabm.0c00638>.

Full materials and methods, low-density cell viability and cell recovery data, flow cytometry forward and side scatter data, flow cytometry isotype controls, flow cytometry mean fluorescence values, light microscope images of thawed cells, and stem cell differentiation into osteoblasts and adipocytes (all samples) (PDF)

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### Author Contributions

M.G. and K.M. devised the experiments and planned the research. K.M. undertook experimental work unless otherwise indicated. R.T. undertook flow cytometry analysis. The manuscript was written by all authors, who have given approval of the final version.

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### Notes

The authors declare the following competing financial interest(s): M.I.G. is a named inventor on a patent application relating to the material used in this article.

Background data is available at [wrap.warwick.ac.uk](http://wrap.warwick.ac.uk).

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