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Description	

**Long-term cryopreservation of human mesenchymal stem cells using carboxylated
poly-L-lysine without the addition of proteins or dimethyl sulfoxide**

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Abstract

Human bone marrow-derived mesenchymal stem cells (hBMSCs) are known for their potential to undergo mesodermal differentiation into many cell types, including osteocytes, adipocytes, and chondrocytes. Therefore, hBMSCs could be used for a variety of regenerative medicine therapies; in fact, hBMC-derived osteocytes have already been used in bone reconstruction. This study discusses the viability and the differentiation properties of hBMSCs that have been cryopreserved in the absence of proteins or dimethyl sulfoxide (DMSO), by using a novel polyampholyte cryoprotectant (CPA). This CPA is based on carboxylated poly-L-lysine (COOH-PLL) and was prepared by a reaction between ϵ -poly-L-lysine and succinic anhydride. $^1\text{H-NMR}$ and two-dimensional correlation ($^1\text{H-}^{13}\text{C}$ HSQC) spectroscopy revealed that COOH-PLL did not have a special structure in solution. hBMSCs can be cryopreserved for 24 months at -80°C by using a 7.5% (w/w) cryopreserving solution of COOH-PLL which introduces carboxyl groups that result in $>90\%$ cell viability after thawing. Furthermore, the cryopreserved hBMSCs fully retained both their proliferative capacity as well as their potential for osteogenic, adipogenic, and chondrogenic differentiation. Confocal laser-scanning microscopy findings showed that the polyampholyte CPA did not penetrate the cell membrane; rather, it attached to the membrane during cryopreservation. These results indicate that the cryoprotective mechanisms of COOH-PLL

might differ from those of currently used small-molecule CPAs. These results also suggest that using COOH-PLL as a cryoprotectant for hBMSC preservation can eliminate the use of proteins and DMSO, which would be safer if these cells were used for cell transplantation or regenerative medicine.

Keywords: cryopreservation, mesenchymal stem cell, polyampholyte, tissue engineering

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent cells that can prove useful in developing regenerative therapies for various mesodermal tissues, including bone, cartilage, tendon, muscle, and blood vessels [1-3]. Human bone marrow-derived MSCs (hBMSCs) and adipose-derived MSCs have already been used clinically for tissue engineering to regenerate fat, vasculature, and bone [4-6]. Successfully freezing hBMCs is critical for their clinical and research use, especially when preserving tissues for future cell transplantation. Since Polge reported that a glycerol solution could preserve cattle sperm at -79°C in 1949 [7], rapid improvement in the science of cryobiology led to the discovery of dimethyl sulfoxide (DMSO) as a cryoprotective agent (CPA) [8]. Although many chemicals are known as CPAs including low and high molecular weight chemicals [9-11], DMSO is quite effective and is used in cell banks worldwide. Many kinds of stem cells can be preserved with DMSO-based cryopreservation solutions [12-14]. However, DMSO is toxic [15,16], and it affects the differentiation of many cell types [17,18]. In addition, it is reported that DMSO has an impact on epigenetic profile in mouse embryoid body [19]. Therefore, it needs to be rapidly eliminated after thawing. Although many studies have successfully cryopreserved MSCs with DMSO, few studies have focused on the development of novel CPAs [12,14]. And also, regulatory concerns of using DMSO as a clinical cryoprotectant is one issue of the driving

the need to develop a cryopreservation solution for stem cells.

In this study, we demonstrated that the carboxyl group introduced by ϵ -poly-L-lysine (COOH-PLL), which is classified as a polyampholyte, has cryoprotective properties [20-23]. In previous studies, many mammalian cell types, including human induced pluripotent stem (iPS) cells [21,22] were well cryopreserved using COOH-PLLs. However, the period of cryopreservation was only 1 week, and long-term preservation was not investigated. Moreover, the detailed mechanisms by which COOH-PLL acts as a cryoprotective agent are still unknown. In this study, we successfully cryopreserved hBMSCs for up to 24 months with COOH-PLL, a non-membrane-penetrating CPA. We then compared cell viability, proliferation, and differentiation activities among non-frozen cells and cells cryopreserved with DMSO or COOH-PLL. We also investigated the mechanisms by which COOH-PLL acts as a cryoprotectant by examining its membrane permeability, to compare it with DMSO.

2. Materials and Methods

2.1 Cell culture

Five independent hBMSCs (HMS0051, HMS0008, HMS0050, HMS0023, HMS0024) deposited by Dr. Kato in Hiroshima University [24] were purchased from the

RIKEN Cell Bank (RIKEN Bioresource Center, Ibaraki, Japan) in accordance with the Life Science Committee of Japan Advanced Institute of Science and Technology, and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS), 3ng/mL basic fibroblast growth factor (bFGF; Wako Pure Chem. Ind. Ltd., Osaka Japan), 100 units/mL penicillin, and 100µg/mL streptomycin. These hBMSCs were delivered in the dry ice from the cell bank and stored in the liquid nitrogen after arriving at our laboratory until using. Cell culture was carried out at 37°C under 5% CO₂ in a humidified atmosphere. When the cells reached 80% confluence, they were detached using 0.25% (w/v) trypsin containing 0.02% (w/v) of ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline without calcium or magnesium (PBS(-)) and seeded on a new tissue culture plate for subculture. The hBMSCs used in this study were at stages between 3 and 5 passages.

2.2 COOH-PLL preparation

COOH-PLL was prepared as described in our previous study [20]. Briefly, a 25% (w/w) ε-poly-L-lysine (PLL, MW 4000) aqueous solution (JNC Corp., Tokyo, Japan) was mixed with succinic anhydride (SA) (Wako) at 0%–100% mol ratios (SA/PLL amino groups) and incubated at 50°C for 1 h to convert amino groups into carboxyl groups. The number of amino groups was determined using the 2,4,6-trinitrobenzenesulfonate (TNBS) method [25].

Briefly, 0.3 mL of 250 $\mu\text{g/mL}$ sample solution, 1 mL of 1.0 mg/mL TNBS solution, and 2 mL of 40 mg/mL sodium bicarbonate aqueous solution containing 10 mg/mL sodium dodecyl sulfate (pH 9.0) were mixed and incubated at 37°C for 2 h. After the mixture was cooled to 25°C, the absorbance was measured at 335 nm. The ratio of carboxylation, which was determined by TNBS assay, shown in parentheses [e.g., PLL (0.65)] indicated that 65% of the α -amino groups have been converted into carboxyl groups by SA addition.

2.3 NMR measurements

The ^1H -NMR spectra of 7.5% COOH-PLL in 90% $\text{H}_2\text{O}/10\%$ D_2O solution were recorded at 25°C on Varian INOVA spectrometer operating at 600 MHz, equipped with a pulse-field gradient triple-resonance probe. The spectral assignments were achieved by using a combination of COSY, TOCSY, HSQC, and HMBC techniques. DIPSI-2 was used for TOCSY mixing, with duration of 35 ms. All the spectra were processed with the NMRPipe program. The NMRViewJ program was employed for spectral analysis.

The ^1H and ^{13}C assignments of the lysine moiety were as follows: (8.43: $\text{HN}\zeta$, $\text{R}_{-1}(\text{a})$), (8.16: $\text{HN}\alpha$, $\text{R}_0(\text{s})$), (8.10: $\text{HN}\zeta$, $\text{R}_{-1}(\text{s})$), (4.10: $\text{H}\alpha$, 57.0: $\text{C}\alpha$, $\text{R}_0(\text{s})$), (3.85: $\text{H}\alpha$, 56.2: $\text{C}\alpha$, $\text{R}_0(\text{a})$), (1.63 and 1.74: $\text{H}\beta$, 33.5: $\text{C}\beta$, $\text{R}_0(\text{s})$), (1.82: $\text{H}\beta$, 33.3: $\text{C}\beta$, $\text{R}_0(\text{a})$; degenerated), (1.31: $\text{H}\gamma$, 25.2: $\text{C}\gamma$, $\text{R}_0(\text{s})$; degenerated), (1.30: $\text{H}\gamma$, 24.3: $\text{C}\gamma$, $\text{R}_0(\text{a})$; degenerated), (1.49: $\text{H}\delta$, 30.6: $\text{C}\delta$; degenerated), (3.13 and 3.17: $\text{H}\epsilon$, 41.5: $\text{C}\epsilon$, $\text{R}_{-1}(\text{s})$), and (3.19 ppm: $\text{H}\epsilon$, 42.0: $\text{C}\epsilon$,

R-1(a); degenerated). The ^1H -peak assignments of succinate are (2.46: H3, 34.8: C3; degenerated) and (2.42: H2, 35.5: C2; degenerated).

2.4 Cryopreservation protocol

The cryopreservation solutions were prepared as follows: COOH-PLLs were dissolved in DMEM without FBS at the concentration of 7.5% (w/w), and the pH was adjusted to 7.4 by using HCl or NaOH. Osmotic pressure was measured by an osmometer (Osmometer 5520; Wescor, Inc. UT, USA) and adjusted to about 600 mOsm in the cryopreservation solution of PLL derivatives by using a NaCl solution. The cells were counted and resuspended in 1 mL of COOH-PLL solution or 10% DMSO culture medium without FBS at a density of 1×10^6 cells/mL in 1.9-mL cryovials (Nalgene, Rochester, NY) and stored in a -80°C freezer without controlling the cooling rate for 1 week or 24 months.

2.5 Cell viability and proliferation assay

Individual vials were thawed in a water bath at 37°C with gentle shaking, after which the thawed cells were diluted in 9 mL of DMEM. After centrifugation at 1000 rpm for 5 min, the supernatant was removed, and the cells were resuspended in 5 mL of medium. The cells were plated in 6-well culture plates at a cell density of $1 \times 10^4/\text{cm}^2$ ($n = 5$). To determine cell survival, the medium including dead float cells was collected, and the

attached cells were trypsinized. All the cells were stained with trypan blue and counted using a hemocytometer immediately post-thawing (0 h) and 6 h later. The reported values are the ratios of living cells to total number of cells. To study cell proliferation after thawing, the cells were seeded onto 24-well culture plates at a cell density of $1.0 \times 10^5/\text{cm}^2$ with 2 mL of DMEM (n = 3). The cell number was counted 24, 48, 72, and 96 h after seeding and was used to calculate the doubling time.

2.6 Fluorescein isothiocyanate (FITC) labeling of PLL

For fluorescent labeling, 25% PLL solution was treated with FITC at a 1/100 molar ratio to PLL for 16h at 50°C. FITC-PLL was purified by dialysis (molecular weight cutoff, 10kDa; Spectra/Por, Spectrum Laboratories, Inc., CA, USA) against water for 48 h. A reaction with SA was performed to obtain FITC-labeled COOH-PLLs. The interaction between FITC-labeled COOH-PLLs and hBMSCs following cryopreservation was observed by confocal laser-scanning microscopy (FLUOVIEW FV500; Olympus, Tokyo, Japan).

2.7 Flow cytometry analysis

Unfrozen and thawed hBMSCs that had been cryopreserved for 24 months by using 7.5% PLL (0.65) medium were incubated with phycoerythrin (PE)-conjugated anti-cluster of differentiation (CD) antibody on ice for 1 h in the dark. After washing, the

cells were loaded in a FACSCalibur flow cytometer (BD Bioscience Immunocytometry Systems, San Jose, CA, USA), and a minimum of 20,000 events were counted. The antibodies used in this experiment were CD13, CD14, CD29, CD34, and CD105 (eBioscience, Inc., San Diego, CA, USA). PE-conjugated mouse immunoglobulin G₁ (IgG₁) (eBioscience) was used as an isotype control.

2.8 Induction of differentiation of hBMSCs

Unfrozen and thawed hBMSCs which had been cryopreserved for 24 months with a 7.5% PLL (0.65) solution, were seeded at a cell density of $1 \times 10^4/\text{cm}^2$ per well in 12-well culture plates. After confluence, the hBMSCs were induced to differentiate into osteoblasts and adipocytes for 14 days in osteogenic and adipogenic media, respectively [26]. Osteogenic medium contained 0.1 μM dexamethasone, 10 mM β -glycerophosphate disodium salt, 0.07 mM L-ascorbic acid 2-phosphate magnesium salt n-hydrate (Sigma), 10% FBS, and 1% antibiotic/antimycotic solution in DMEM. Adipogenic medium contained 0.5 mM isobutyl methylxanthine, 0.1 mg/mL insulin, 0.2 mM indomethacin, 1 μM dexamethasone, 10% FBS, and 1% antibiotic/antimycotic solution in DMEM. The culture medium was changed 3 times per week. Chondrogenic differentiation was performed in a pellet culture [27]. Briefly, approximately 2.5×10^5 of unfrozen and cryopreserved hBMSCs were centrifuged at $300 \times g$ for 4 min in a conical tube to produce a mass pellet. The pellet was cultured at 37°C with

CO₂ for 14 days in 500 µL of chondrogenic differentiation medium containing 6.25 µg/mL insulin, 6.25 µg/mL transferrin, 6.25 µg/mL selenious acid, 1.25 mg/mL bovine serum albumin, 5.35 mg/mL linoleic acid, 0.07 mM L-ascorbic acid 2-phosphate magnesium salt n-hydrate, 0.1 µM dexamethasone, 10 ng/mL tumor growth factor-β1 (TGF-β1), 10% FBS, and 1% antibiotic/antimycotic solution in DMEM. The medium was replaced every 3 days. For all negative controls, the cells were maintained in DMEM containing 10% FBS.

2.9 Evaluation of differentiation

Monolayer cultures treated with an osteogenic differentiation medium were stained with 1% Alizarin Red S for 15 min after fixation with 4% neutral buffered formalin for mineralized matrix deposition. Alkaline phosphatase activity was also examined by the modified Kind–King method [28] using a commercially available kit (ALP Kainos kit; Kainos Lab., Inc., Tokyo, Japan). Monolayer cultures treated with adipogenic differentiation medium were stained with oil red O solution to visualize the formation of fat vacuoles. Briefly, the cells were fixed with 4% neutral buffered formalin and incubated for 20 min with 0.2% oil red O isopropanol solution. In addition, glycerol-3-phosphate dehydrogenase (GPDH) activity was determined for the differentiation of adipocytes by using a GPDH assay kit (GPDH assay kit; Primary Cell Co., Ltd., Hokkaido, Japan). Cell pellets for chondrogenic differentiation were fixed in 4% neutral buffered formalin for 1 h, embedded in paraffin, cut

into 3–5- μ m sections, and stained with Alcian blue (0.05% Alcian blue in 3% acetic acid). Cells were also stained with an anti-collagen type II antibody (sc-52658; Santa Cruz Biotechnology, Inc. CA. USA) to detect collagen Type II. In addition, the glycosaminoglycan (GAG) contents in the cell pellets were determined using a dimethylmethylene blue (DMMB) dye-binding assay kit (Blyscan kit; Biocolor Ltd., Newtownabbey, Northern Ireland), according to the manufacturer's instructions.

2.9 Statistical analysis

All data are expressed as the mean \pm standard deviation (SD). The Student's *t*-test was used to make comparisons between 2 groups. A one-tailed Tukey–Kramer test was used to compare data with more than 3 groups. *P* values of <0.05 were considered statistically significant.

3. Results

3.1 COOH-PLL preparation

Figure 1 shows the aliphatic region of ^1H -NMR spectra of COOH-PLL with a carboxylation ratio of (a) 0, (b) 0.4, (c) 0.65, and (d) 0.8. The PLL (0) used in these experiments had an average of 32 lysine residues, and each proton in all the residues (except one at the C-terminus) was observed as a single peak, indicating that the lysine residues do

not form a unique structure, but possess a highly flexible conformation. These results were reflected in the NMR spectral data where each proton from the succinylated lysine residue gave a new peak without a sequence-specific variation in chemical shift. Collectively, no unique structure is induced by the introduction of succinylate to PLL under these conditions. These charts also show that the carboxylation ratios determined by the TNBS assay are consistent with the ratios obtained from $^1\text{H-NMR}$, and that the values are proportionate to the addition of SA.

NMR signals were analyzed by the combined use of COSY, TOCSY, HSQC, and HMBC techniques. These results are summarized in Figure 2. The proton and carbon chemical shifts were mainly affected by succinylation at its own residue (R_0) and the nearest neighboring residue at the N-terminal side (R_{-1}) (Figure 2 (A)). This means that the each signal should split into four signals originating from four different possible structures, namely, $\text{R}_{-1}(\text{a})\text{R}_0(\text{a})$, $\text{R}_{-1}(\text{s})\text{R}_0(\text{a})$, $\text{R}_{-1}(\text{a})\text{R}_0(\text{s})$, and $\text{R}_{-1}(\text{s})\text{R}_0(\text{s})$. In the actual spectrum, however, the carboxylation at R_{-1} caused only a minor effect to the chemical shift of the nuclei in the far side, like the α -, β -, γ -, and δ -nuclei. The change at R_0 did not profoundly affect the chemical shift perturbation to the δ - and ϵ -nuclei as well. Therefore, the nearest substitution has a major contribution to the change in chemical shift, and this is depicted in Figure 2 (B, C). Close inspection of Figure 2 (C) shows that quite a few peaks have a fine structure, which

reflects the minor effects from distant positions, although it is hard to determine from where it is exerted.

3.2 Cell properties after cryopreservation

Figure 3 shows the hBMSCs cell viability immediately and 6 h after thawing, when the cells were cryopreserved for 1 week with 7.5% PLL solution in which various amounts of carboxyl groups were introduced. The results suggest that COOH-PLLs with a ratio of carboxylation of 0.5 to 0.8 have high cryopreservation efficiency. These results agree with the previous results obtained with other PLL cryopreserved cells. Figure 4(A) and 4(B) showed the proliferation curves of hBMSCs after thawing and the doubling time of unfrozen and 24-month cryopreserved cells. Cell viability immediately after 24 months of cryopreservation is shown in Figure 4(C). These results indicate that long-term cryopreservation with PLL (0.65) did not affect the proliferation and viability of hBMSCs.

To determine cell membrane permeability, cells were cryopreserved with FITC-labeled PLL (0.65), after which the absorption between PLL (0.65) and the cell membrane was observed using a confocal laser microscope. PLL (0.65) molecules appeared to be adsorbed to cell membranes immediately after thawing (Figure 5), although no cell membrane penetration was observed.

3.3 Cell-surface markers on cryopreserved hBMSCs

Expression profiles of cell-surface markers on non-frozen cells and cells that had been cryopreserved for 24 months were analyzed using flow cytometry. As Figure 6 clearly shows, the frozen cells were strongly positive for CD13, CD29, and CD105, all of which are commonly used in the analyses of MSCs [29]. Frozen cells were also found to be negative for the hematopoietic cell marker CD14 and the hematopoietic stem cell marker CD34 [30]. The data also revealed no difference between non-frozen and PLL cryopreserved cells (0.65), indicating that the cryoprotectant would not affect the biological properties of the hBMSCs.

3.4 Differentiation of cryopreserved hBMSCs

The results evaluating the differentiation potency after cryopreservation with PLL (0.65) are shown in Figure 7. Histological evaluation revealed that hBMSCs cryopreserved with 7.5% PLL (0.65) for 24 months at $-80\text{ }^{\circ}\text{C}$ successfully differentiated into osteoblasts (Figure 7A), adipocytes (Figure 7B), and chondrocytes (Figure 7C and D). Furthermore, quantitative analyses confirmed that hBMSCs cryopreserved with PLL (0.65) maintained their differentiation properties. Alkaline phosphatase activity was used as a measure for osteoblastic differentiation (Figure 7E), GPDH activity for adipose differentiation (Figure 7F), and the amount of glycosaminoglycan for chondrogenic differentiation (Figure 7G). These results show that each type of differentiation depended on the induction medium used,

and no inappropriate differentiation occurred following cryopreservation with PLL (0.65).

4. Discussion

In this study, we focused on the development of a novel polymeric CPA, COOH-PLL, as an alternative for the cryopreservation of hBMSCs with DMSO and/or exogenous proteins. This study helps validate the use of COOH-PLL as a CPA and provides evidence that it can be used to cryopreserve hBMSCs that may then undergo successful differentiation. COOH-PLL may be an alternative CPA for the cryopreservation of hBMSCs to be used for clinical application.

MSCs have a multipotent ability to differentiate into various functional mesodermal tissue cell types. Although bone marrow- and adipose-derived stem cells that are cryopreserved using DMSO retain their viability and differentiation properties [12,31], DMSO should be avoided because of its toxicity and potential influence on stem cell differentiation. Furthermore, most cryopreservation methods utilize FBS [32]. FBS might play an important role as an osmotic pressure buffer and as a cell membrane protector, but its animal-derived proteins should be avoided in clinical use because of the risk of infection. In this study, hBMSCs were cryopreserved with a simple method of cryopreservation by slow cooling with COOH-PLL as a CPA, without the addition of any other CPAs or animal-derived proteins. The results show that these novel CPAs did not alter the phenotypic characteristics

of hBMSCs, and preserved their viability and proliferative ability after thawing, even after long-term storage (24 months) at -80°C (Figure 4). In addition, cell-surface marker expression (Figure 6) and differentiation properties (Figure 7) were also preserved. Clinically, it is very important that preservation at -80°C up to 24 months does not affect the biological properties of hBMSCs because of the non-requirement of liquid nitrogen and the strict management of temperature.

To characterize the mechanism by which PLL acts as a cryoprotectant, we compared the properties of COOH-PLL with those of other traditionally used cryoprotectant. When the cells are cryopreserved, freezing-induced damage can occur through the formation of intracellular ice, which can lead to cell death [33]. Generally, cell membrane-permeable CPAs have to be added to avoid the freezing damage. DMSO and polyols are membrane-permeable CPAs whose mechanisms of cryopreservation have been actively studied. DMSO protects cells from the intracellular ice formation-induced damage by inducing broadening of the glass transition of water, which may reduce the probability of its nucleation and the subsequent ice formation upon cooling [34]. However, as Figure 5 clearly showed, COOH-PLL cannot penetrate the cell membrane because of its relatively high molecular weight (about 5,000Da) and high charge density. This means that the mechanism by which COOH-PLL acts as a cryoprotective agent probably differs from that of DMSO.

There are other membrane-non-permeable CPAs such as trehalose. Our previous study showed that cell viability after cryopreservation with trehalose was significantly lower than that with DMSO or PLL (0.65) [21]. Toner et al. [35] showed that the introduction of trehalose into the cell via a genetically engineered pore-forming protein resulted in significant improvement in the viability of both cryopreserved and desiccated mammalian cells. Trehalose was also reported to play important roles in water replacement and intracellular glass formation [36]. These results indicate that trehalose is an intracellular CPA, and its addition to the extracellular medium was not effective in cryopreservation because of its low membrane permeability. These results also suggest that the cryoprotective mechanisms of PLL (0.65) are not similar to those of trehalose. Also non penetrating high molecular weight CPAs such as hydroxymethyl starch (HES) were reported [9]. The mechanisms of the cryoprotective property of HES would have something in common to those of polyampholytes. From the previous study, as HES attracts and absorbs water, its viscosity is reduced and the rate of dehydration increases, which then allows cells to be cooled rapidly avoiding intracellular ice crystal formation as well as chilling injury. However, HES showed various cryopreservation property which depends on the cell lines, for example, HES showed high cryoprotective property against red blood cells but was used for nucleated cells cryopreservation in combination with other cryoprotectants [10].

In the literature, many proteins that protect cells from the damage induced during freezing or desiccation have been cited [37,38]. Although the mechanisms of protection are still unknown, a relationship may exist between the structure of a CPA and its ability to protect the cell membrane [39,40]. The NMR results (Figure 1,2) showed that PLL (0.65) did not have any steric structure at room temperature; instead, it had a randomly coiled structure. However, this polymer adsorbed to the cell surface during freezing (Figure 5), which may have then protected the cell membrane. The relationship between the structure of COOH-PLL and its ability to protect the cell membrane during freezing should be investigated in future research.

Cell membrane protection is insufficient in explaining the cryoprotective effects of COOH-PLL, because some mechanism that can avoid the formation of the intracellular ice causing lethal damage to cells during the freezing process has to exist. To cryopreserve cells for long-term storage (such as 24 months at -80°C), intracellular ice formation has to be avoided. During freezing, the increasing osmotic pressure due to the concentrated partially frozen extracellular solution dehydrates the cell [41]. The speed and degree of dehydration both influence cryoprotection [42]. If COOH-PLLs interact with salt molecules, which are concentrated during freezing, the degree of dehydration would be controlled. The appropriate level of dehydration would therefore result in the avoidance of intracellular ice

formation. Future work aimed at investigating the interactions between polyampholytes and salts, polyampholytes and water, and polyampholytes and lipid membranes during freezing, by using spectroscopic methods such as solid-state NMR, will help to elucidate the mechanisms by which COOH-PLLs cryopreserve cells.

Conclusions

We developed a novel polymeric CPA, COOH-PLL, which has effective cryopreservative properties on hBMSCs, without using DMSO or exogenous proteins. COOH-PLLs offer high viability and do not affect the biological properties of the cells even after 24 months of cryopreservation at -80°C . The protective mechanisms of COOH-PLL might differ from those of currently used CPAs. Although thorough analysis of its mechanism of action is needed, COOH-PLL could be an alternative CPA for the cryopreservation of hBMSCs for clinical application.

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Disclosures

Authors have no conflicts of interest.

References

- [1] Caplan AI, Bruder SP. Mesenchymal stem cells: Building blocks for molecular medicine in 21st century. *Trends. Mol. Med.* 2001;7:259-264.
- [2] Miyahara Y, Nagaya N, Kataoka M, Yanagawa B, Tanaka K, Hao H, et al. Monolayered mesenchymal stem cells repaired myocardium after myocardial infarction. *Nat. Med.* 2006;12:459-465.
- [3] Le Douarin, NM, Houssaint E, Jotereau FV, Belo M. Origin of hemopoietic stem cells in embryonic bursa of Fabricius and bone marrow studied through interspecific chimeras. *Proc. Natl. Acad. Sci. USA.* 1975;72:2701-2705.
- [4] Cowan CM, Shi YY, Aalami OO, Chou YF, Mari C, Thomas R, et al. Adipose-derived adult stromal cell heal critical-size mouse calvarial defects. *Nat. Biotechnol.* 2004;22:560-567.
- [5] Matsumoto D, Sato K, Gonda K, Takaki Y, Shigeura T, Sato T, et al. Cell-assisted lipotransfer: supportive use of human adipose-derived cells for soft tissue augmentation with lipoinjection. *Tissue Eng.* 2006;12:3375-3382.
- [6] Wiren KM, Hashimoto JG, Semirale AA, Zhang XW. Bone vs. fat: embryonic origin of progenitors determines response to androgen in adipocytes and osteoblasts. *Bone.* 2011;49:662-672.
- [7] Polge C, Smith AU, Parkes AS. Revival of spermatozoa after vitrification and

dehydration at low temperatures. *Nature*. 1949;164:666.

[8] Lovelock JE, Bishop MW. Prevention of freezing damage to living cells by dimethyl sulphoxide. *Nature*. 1959;183:1394-1395.

[9] Stolzing A, Naaldijk Y, Fedorova V, Sethe S. Hydroxyethylstarch in cryopreservation – Mechanisms, benefits and problems. *Transfus. Apher. Sci.* 2012;46:137-147.

[10] Stiff PJ, Murgu AJ, Zaroulis CG, DeRisi MF, Clarkson BD. Unfractionated human marrow cell cryopreservation using dimethylsulfoxide and hydroxyethyl starch. *Cryobiology* 1983;20:17–24.

[11] Thirumala S, Gimble JM, Devireddy RV. Cryopreservation of stromal vascular fraction of adipose tissue in a serum-free freezing medium. *J. Tissue Eng. Regen. Med.* 2010;4:224-232.

[12] Kotobuki N, Hirose M, Machida H, Katou Y, Muraki K, Takakura Y, Ohgushi H. Viability and osteogenic potential of cryopreserved human bone marrow-derived mesenchymal cells. *Tissue Eng.* 2005;11:663-673.

[13] Petrenko YA, Jones DRE, Petrenko AY. Cryopreservation of human fetal liver hematopoietic stem/progenitor cells using sucrose as an additive to the cryopreservation medium. *Cryobiology*. 2008;57:195-200.

[14] Oishi K, Noguchi H, Yukawa H, Miyazaki T, Kato R, Kitagawa Y, et al.

Cryopreservation of mouse adipose tissue-derived stem/progenitor cells. *Cell Transplant.* 2008;17:35-41.

[15] Lowenthal RM, Park DS, Goldman JM, Hill RS, Whyte G, Th'ng KH. The cryopreservation of leukaemia cells: morphological and functional changes. *Br. J. Haematol.* 1976;34:105-117.

[16] Douay L, Gorin NC, David R, Stachowiak J, Salmon C, Najman A, Duhamel G. Study of granulocyte-macrophage progenitor (CFUc) preservation after slow freezing of bone marrow in the gas phase of liquid nitrogen. *Exp. Hematol.* 1982;10:360-366.

[17] Jiang GS, Bi KH, Tang TH, Wang JW, Zhang YK, Zhang W, et al. Down-regulation of TRRAP-dependent hTERT and TRRAP-induced CAD activation by Myc/Max contributes to the differentiation of HL60 cells after exposure to DMSO. *Int. Immunopharmacol.* 2006;6:120413.

[18] Katkov II, Kim MS, Bajpai R, Altman YS, Mercola M, Loring JF, et al. Cryopreservation by slow cooling with DMSO diminished production of Oct-4 pluripotency marker in human embryonic stem cells. *Cryobiology.* 2006;53:194-205.

[19] Iwatani M, Ikegami K, Kremenska Y, Hattori N, Tanaka S, Yagi S, et al. Dimethyl Sulfoxide Has an Impact on Epigenetic Profile in Mouse Embryoid Body. *Stem Cells.* 2006;24:2549-2556.

- [20] Matsumura K, Hyon SH. Polyampholytes as low toxic efficient cryoprotective agents with antifreeze protein properties. *Biomaterials*. 2009;30:4842-4849.
- [21] Matsumura K, Bae JY, Hyon SH. Polyampholytes as cryoprotective agents for mammalian cell cryopreservation. *Cell Transplant*. 2010;19: 691-699.
- [22] Matsumura K, Bae JY, Kim HH, Hyon SH. Effective vitrification of human induced pluripotent stem cells using carboxylated ϵ -poly-L-lysine. *Cryobiology*. 2011;63:76-83.
- [23] Vrana N, Matsumura K, Hyon SH, Geever L, Kennedy J, Lyons J, et al. Cell encapsulation and cryostorage in PVA/gelatin cryogels: Incorporation of carboxylated ϵ -poly-L-lysine as cryoprotectant. *J. Tissue. Eng. Regen. Med*. 2012;6:280-290.
- [24] Tsutsumi S, Shimazu A, Miyazaki K, Pan H, Koike C, Yoshida E, et al. Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF. *Biochem. Biophys. Res. Commun*. 2001;288:413-419.
- [25] Habeeb AF. Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Anal. Biochem*. 1966;14:328-336.
- [26] Liu TM, Martina M, Hutmacher DW, Hui JHP, Lee EH, Lim B. Identification of common pathways mediating differentiation of bone marrow- and adipose tissue-derived human mesenchymal stem cells into three mesenchymal lineages. *Stem Cells*. 2007;25:750-760.
- [27] Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO, Pittenger MF.

Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow.

Tissue Eng. 1998;4:415-428.

[28] Kind PRN, King EJ. Estimation of plasma phosphatase by determination of hydrolysed phenol with amino-antipyrine. J. Clin. Pathol. 1954;7:322-326.

[29] Ishige I, Nagamura-Inoue T, Honda MJ, Harnprasopwat R, Kido M, Sugimoto M, et al. Comparison of mesenchymal stem cells derived from arterial, venous, and Wharton's jelly explants of human umbilical cord. Int. J. Hematol. 2009;90:261-269.

[30] Yang J, Song T, Wu P, Chen Y, Fan X, Chen H, Zhang J, Huang C. Differentiation potential of human mesenchymal stem cells derived from adipose tissue and bone marrow to sinus node-like cells. Mol. Med. Report. 2012;5:108-113.

[31] Gonda K, Shigeura T, Sato T, Matsumoto D, Suga H, Inoue K, et al. Preserved proliferative capacity and multipotency of human adipose-derived stem cells after long-term cryopreservation. Plast. Reconstr. Surg. 2008;121:401-410.

[32] Men H, Agca Y, Critser ES, Critser JK. Beneficial effects of serum supplementation during in vitro production of porcine embryos on their ability to survive cryopreservation by open pulled straw vitrification. Theriogenology. 2005;64:1340-1349.

[33]. Mazur P. The role of intracellular freezing in the death of cells cooled at supraoptimal rates. Cryobiology. 1977;14:251-272.

- [34] Mandumpal JB, Kreck CA, Mancera RL. A molecular mechanism of solvent cryoprotection in aqueous DMSO solutions. *Phys. Chem. Chem. Phys.* 2011;13:3839-3842.
- [35] Eroglu A, Russo MJ, Bieganski R, Fowler A, Cheley S, Bayley H, et al. Intracellular trehalose improves the survival of cryopreserved mammalian cells. *Nat. Biotechnol.* 2000;18:163-167.
- [36] Crowe JH, Crowe LM, Tablin F, Wolkers WF, Oliver AE, Tsvetkova N. Stabilization of cells during freeze-drying; The trehalose myth. In: Fuller BJ, Lane N, Benson EE, editors, *Life in the Frozen State*, Boca Raton: CRC Press; 2004, p 3-65.
- [37] Thalhammer A, Hundertmark M, Popova AV, Seckler R, Hinch DK. Interaction of two intrinsically disordered plant stress proteins (COR15A and COR15B) with lipid membranes in the dry state. *Biochim. Biophys. Acta.* 2010;1798:1812-1820.
- [38]. Thomashow MF. Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 1999;50: 571-599.
- [39] Shimizu T, Kanamori Y, Furuki T, Kikawada T, Okuda T, Takahashi T, et al. Desiccation-induced structuralization and glass formation of group 3 late embryogenesis abundant protein model peptides. *Biochemistry.* 2010;16:1093-1104.
- [40] Popova AV, Hundertmark M, Seckler R, Hinch DK. Structural transitions in the intrinsically disordered plant dehydration stress protein LEA7 upon drying are modulated

by the presence of membranes. *Biochim. Biophys. Acta.* 2011;1808:1879-1887.

[41] Mazur P, Seki S, Pinn IL, Kleinhans FW, Edashige K. Extra- and intracellular ice formation in mouse oocytes. *Cryobiology.* 2005;51:29-53.

[42] Mazur P, Koshimoto C. Is intracellular ice formation the cause of death of mouse sperm frozen at high cooling rates? *Biol. Reprod.* 2002;66:1485-1490.

Figure Legends

Figure 1

¹H-NMR spectra of COOH-PLL with (A) 0, (B) 0.4, (C) 0.65, and (D) 0.8 molar ratio of introduced carboxyl groups

Figure 2

NMR signals analyzed by the combined use of COSY, TOCSY, HSQC, and HMBC techniques

(A) Chemical structure of COOH-PLL. (B) Summary of signal assignment of PLL (0.65). (C) ¹H-¹³C HSQC spectrum of COOH-PLL (0.65). Each lysine proton has two sets of HSQC signals from the succinate-free and succinylated structures. Plotted with NMRViewJ 8.0 software (One Moon Scientific, Inc.).

Figure 3

Viability of hBMSCs after cryopreservation for 1 week with 10% DMSO and 7.5% (w/w) PLL with different ratios of introduced COOH

Cell viability immediately (white bars), and 6 h (gray bars) after thawing at 37°C. Data are expressed as the mean ± SD for 5 independent experiments.

Figure 4

(A) Growth curves of hBMSC cells, unfrozen control (open circles), cryopreserved with 10%

DMSO (open triangles), 7.5% PLL(0.65) (open squares) for 96 h (n = 3). Data are expressed as mean \pm SD for 3 independent experiments. (B) Doubling time of unfrozen hBMSCs and hBMSCs cryopreserved with either 10% DMSO or 7.5% PLL (0.65) for 24 months and (C) viability of hBMSCs after cryopreservation with either 10% DMSO or 7.5% PLL (0.65) for 24 months. Data are expressed as the mean \pm SD for 5 independent experiments. NS, no significant difference.

Figure 5

Confocal microscopy images of hBMSCs cryopreserved with FITC-labeled PLL (0.65)

(A) Dark and (B) bright fields at the same point. Bar, 10 μ m.

Figure 6

Representative data showing cell-surface marker expression in non-frozen cells and cells cryopreserved with 7.5% PLL (0.65) for 24 months as analyzed by flow cytometry

The x-axis indicates fluorescence intensity, and the y-axis, cell counts. The dotted and solid lines indicate the results obtained using the isotype control or specific antibodies, respectively.

Figure 7

Differentiation of hBMSCs after cryopreservation for 24 months

(A-D) Histological evaluation of the differentiation of hBMSCs cryopreserved with PLL

(0.65). Bar, 100 μm . (A) Alizarin red staining for osteoblast differentiation after induction culture, (B) oil red O staining for adipocyte differentiation after induction culture, and (C, D) Alcian blue staining and collagen type II immunostaining for chondrocyte differentiation, after induction culture. (E-G) Quantitative analyses of the differentiation of hBMSCs. (E) alkaline phosphatase activity, (F) GPDH activity, and (G) amounts of GAG in induced and uninduced cultures of unfrozen and cryopreserved hBMSCs. Data are expressed as the mean \pm SD for 5 independent experiments. NS, no significant difference, *** $P < 0.001$.

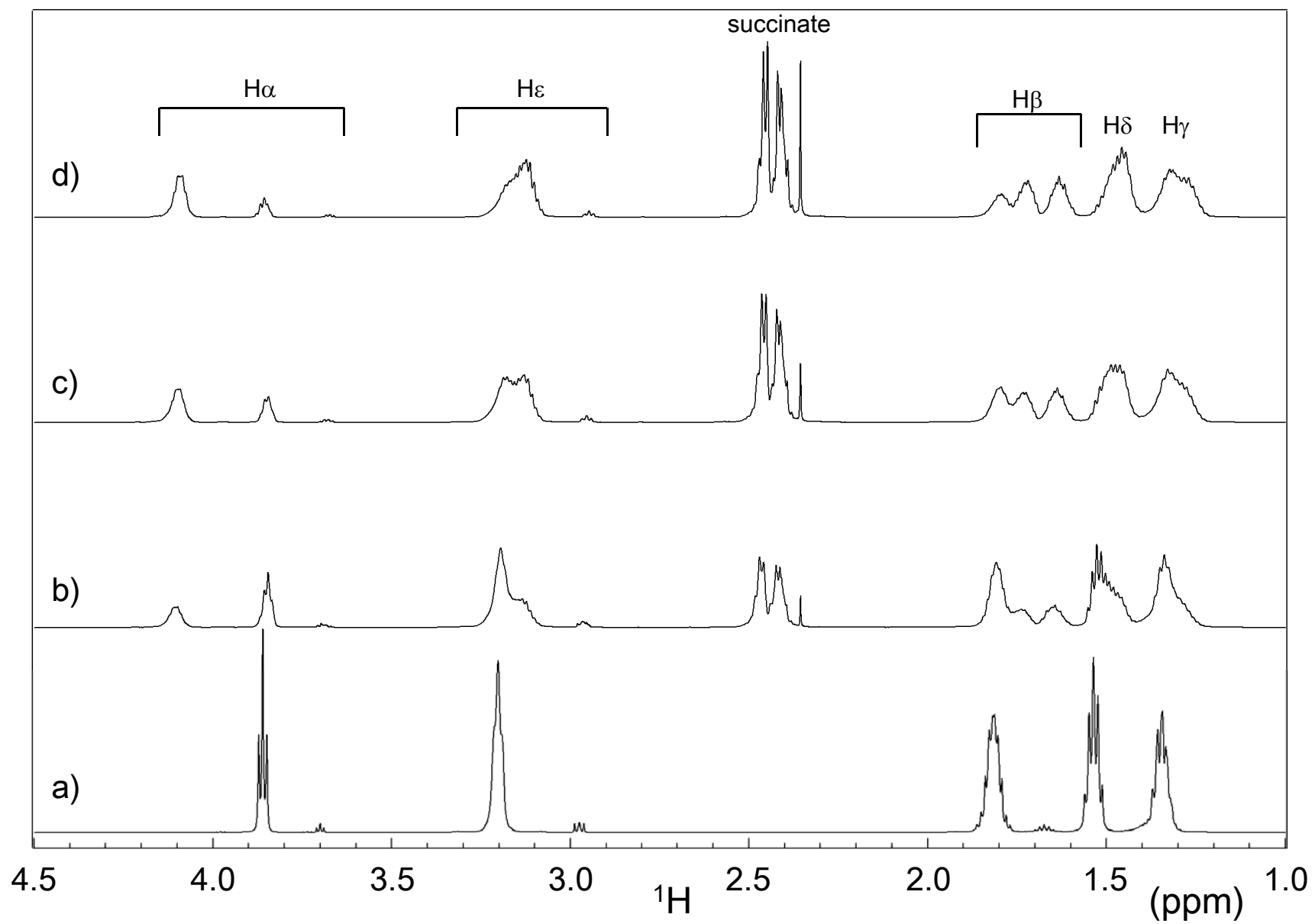


Fig1

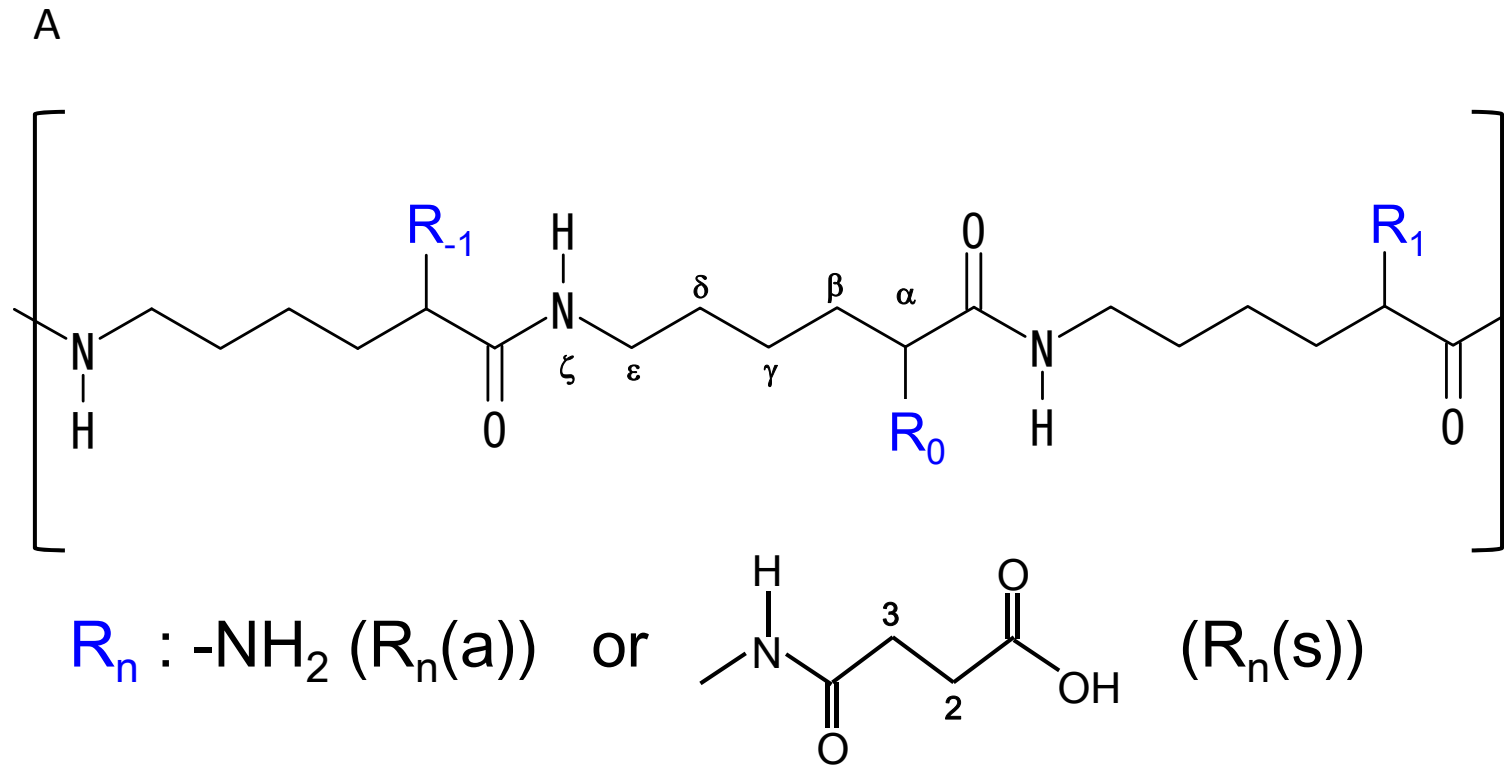


Fig2(A)

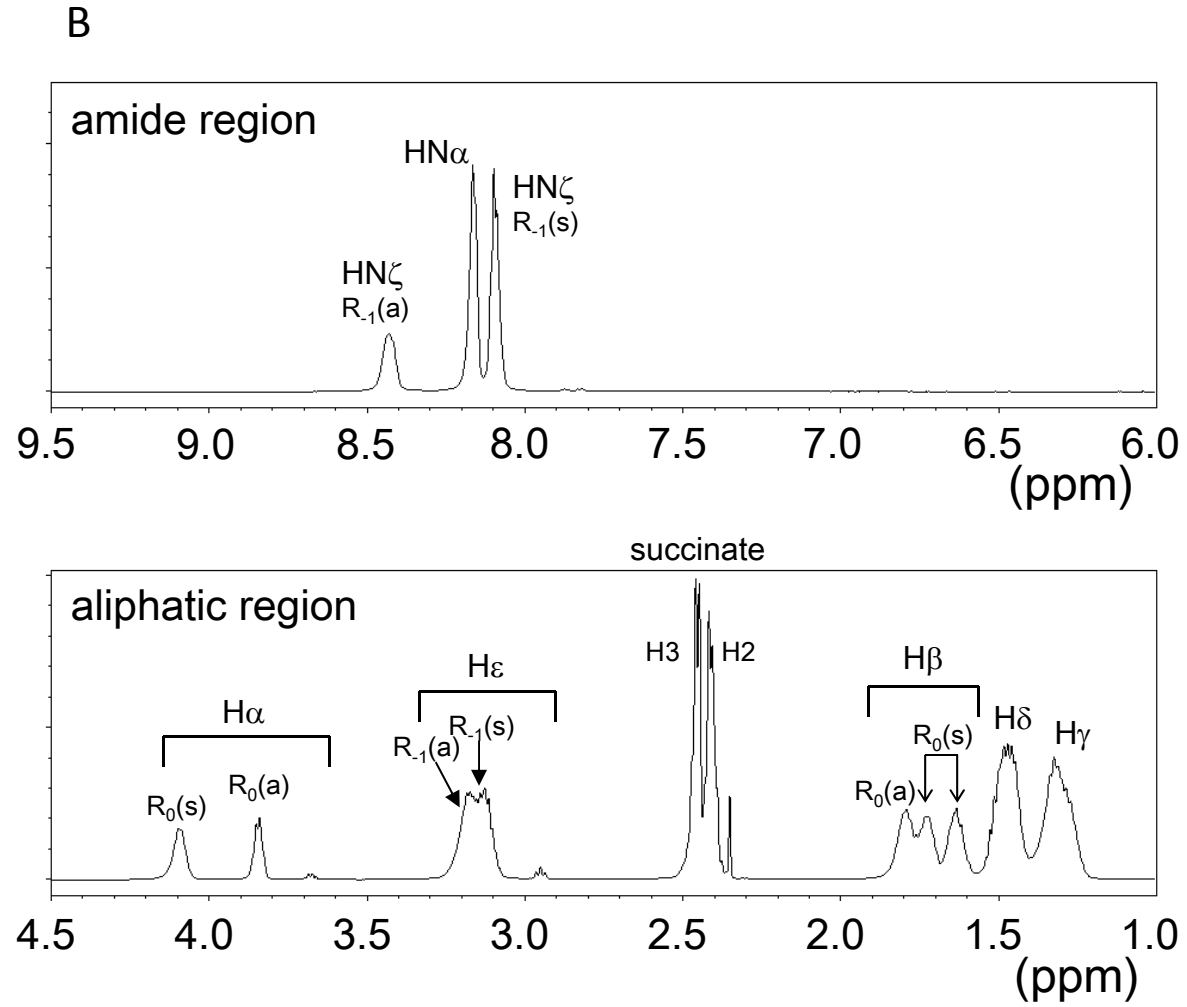


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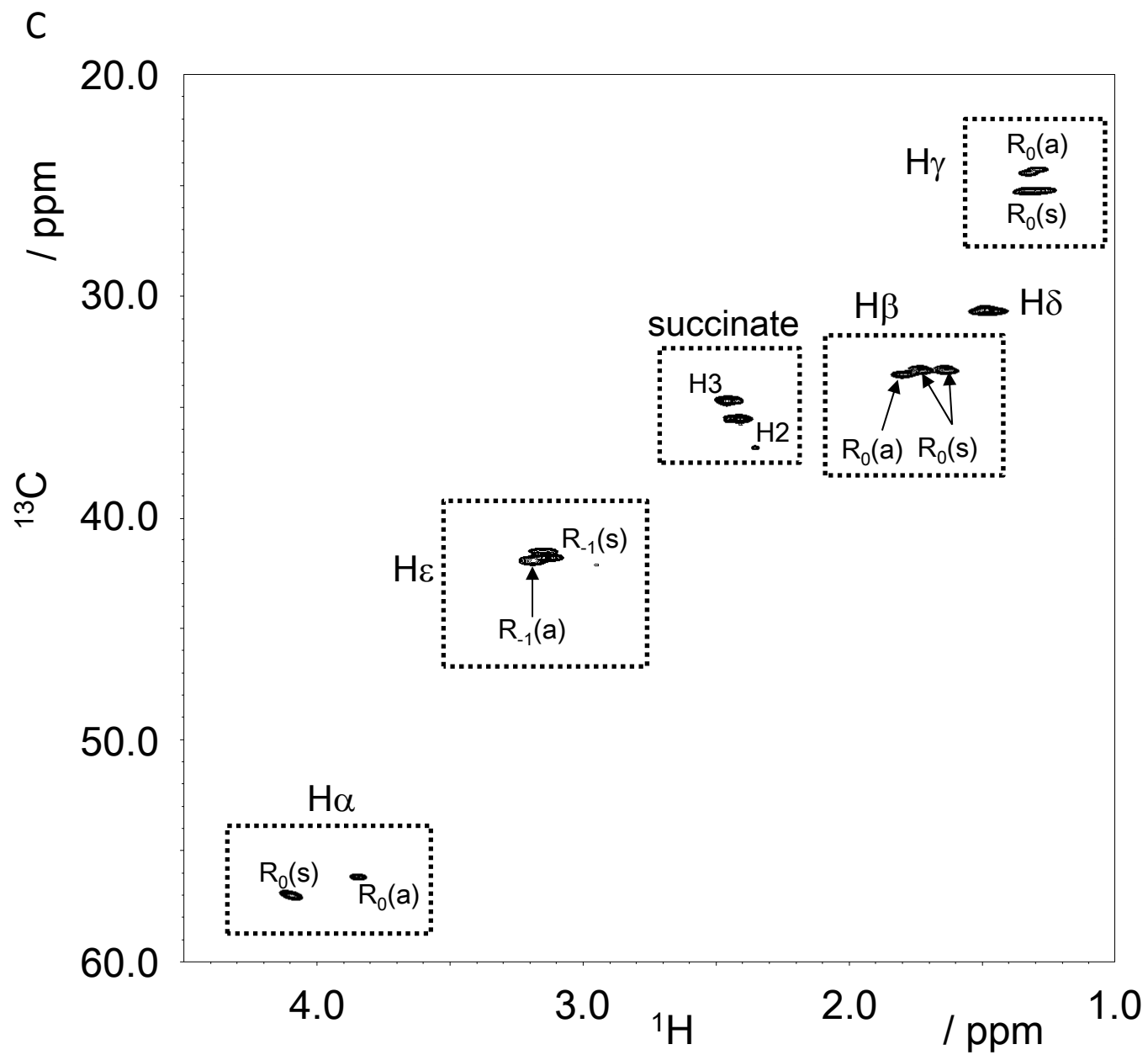


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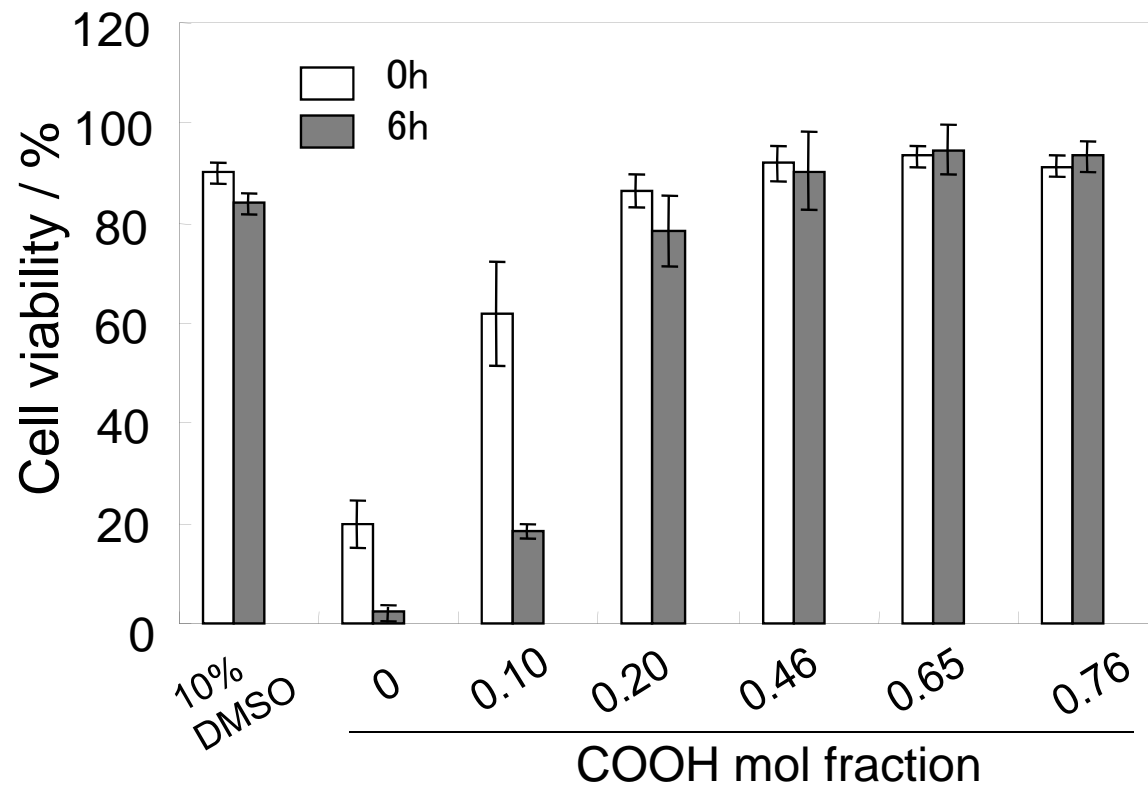


Fig3

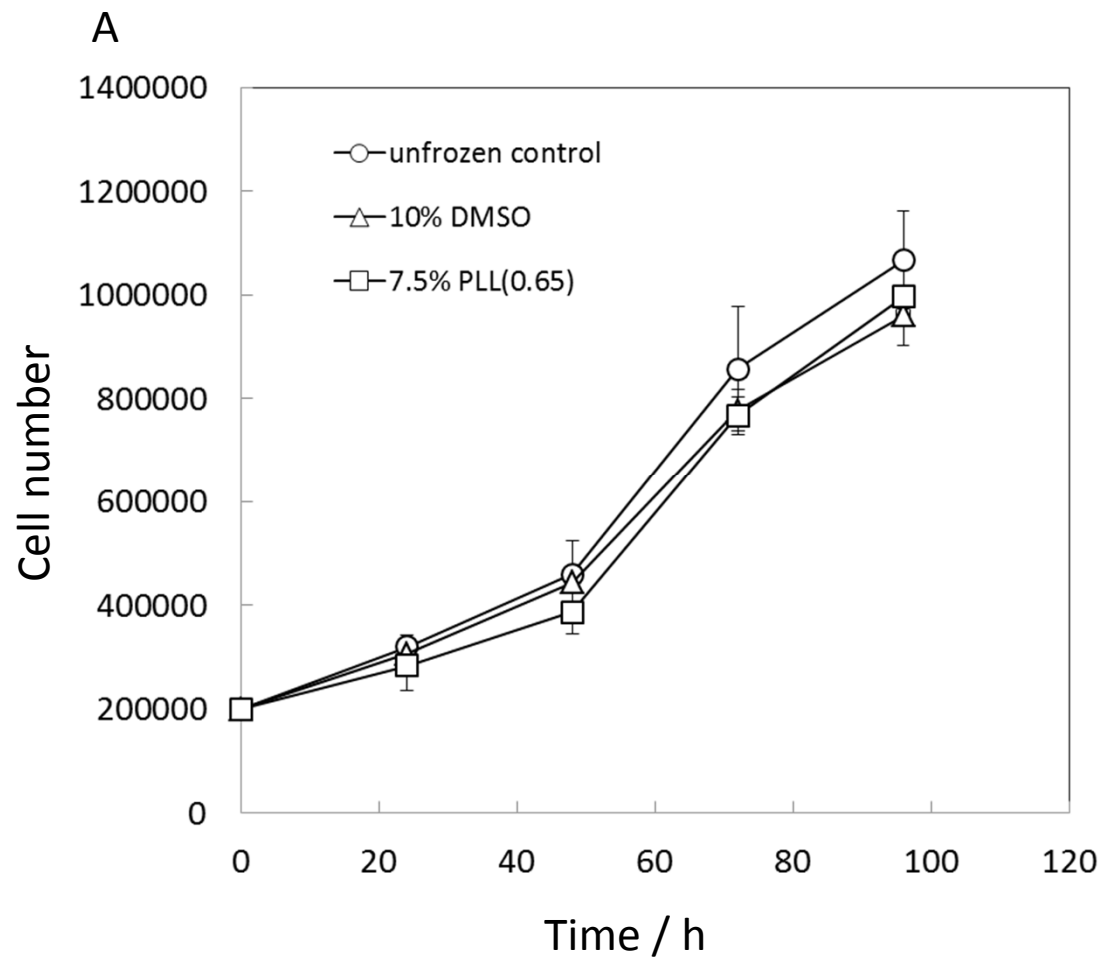


Fig4 (A)

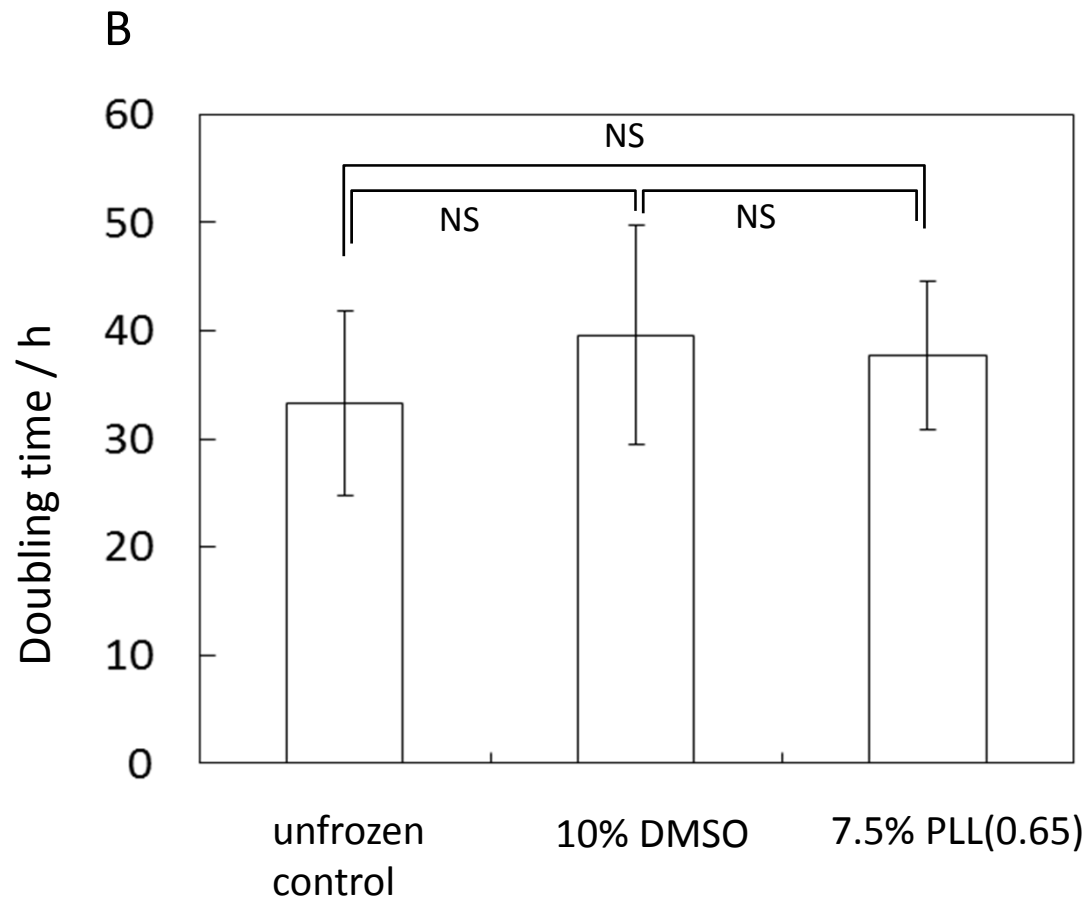


Fig4 (B)

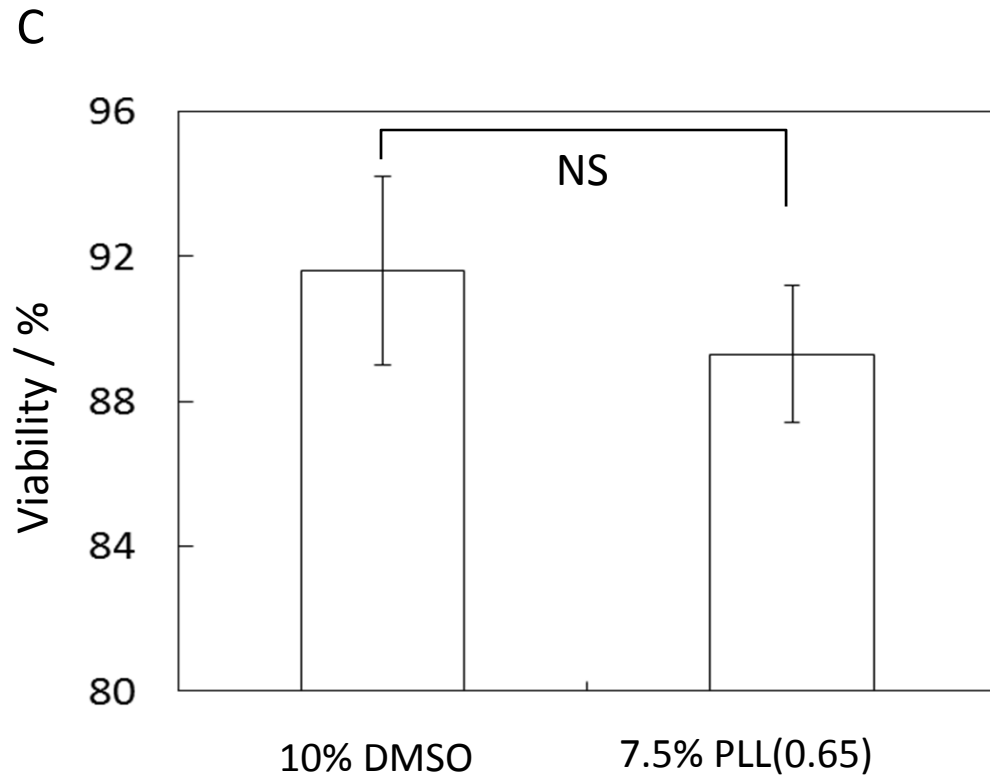


Fig4 (C)

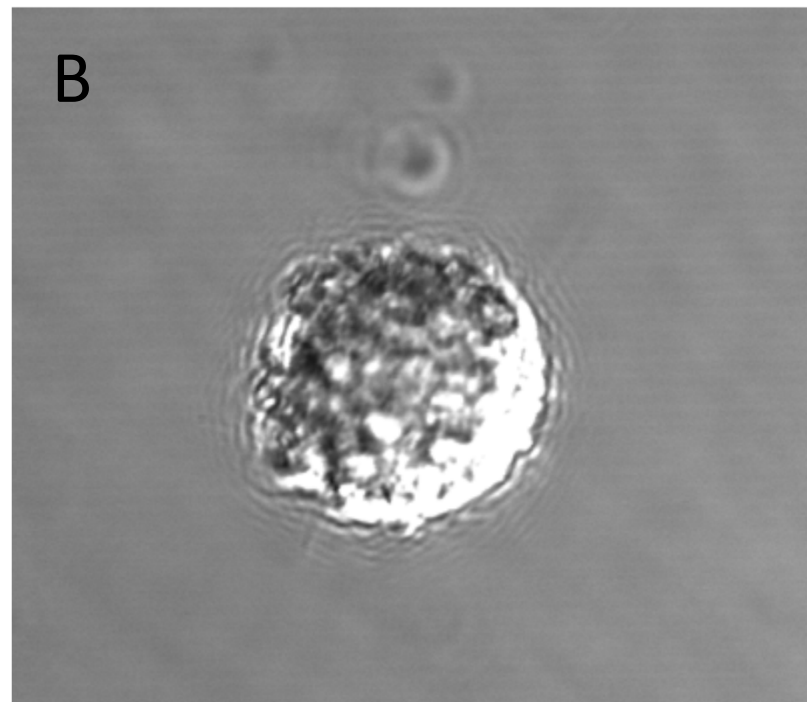
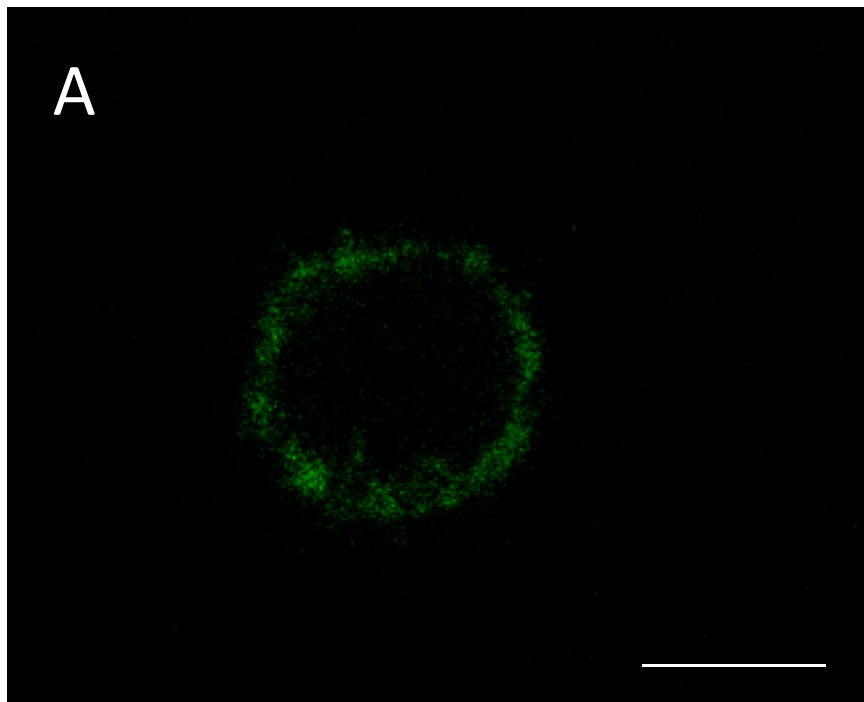
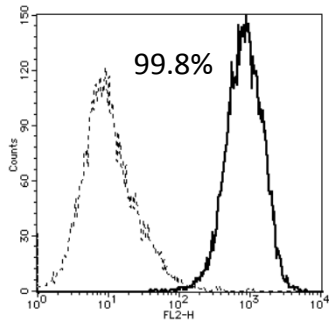
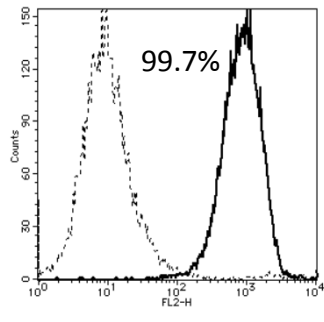


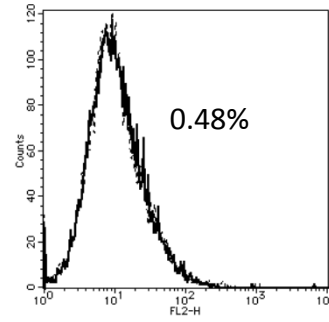
Fig5



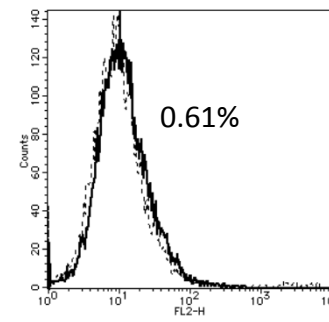
CD13 unfrozen



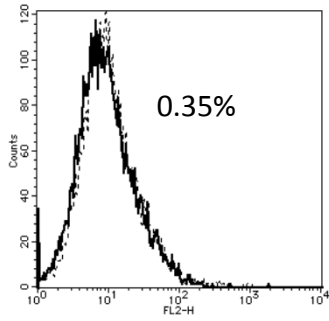
CD13 PLL(0.65)



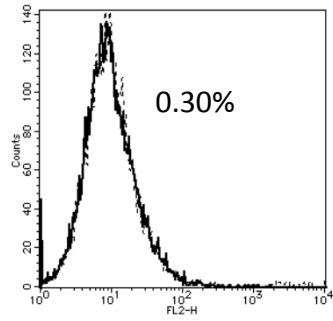
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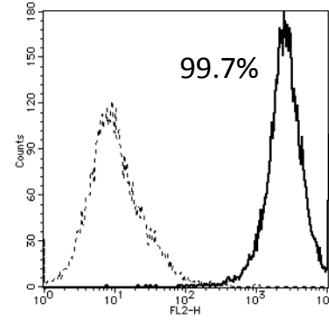
CD34 PLL(0.65)



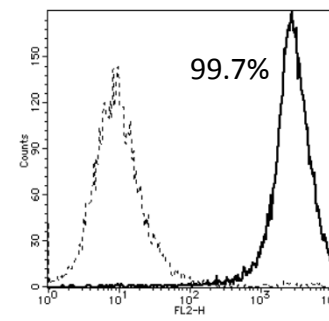
CD14 unfrozen



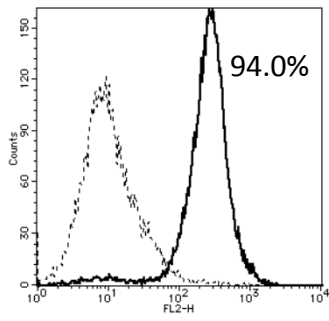
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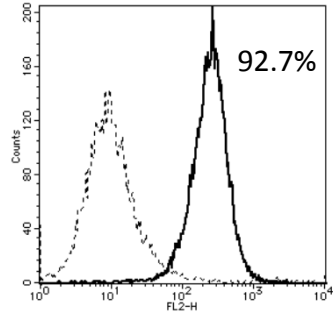
CD105 unfrozen



CD105 PLL(0.65)



CD29 unfrozen



CD29 PLL(0.65)

Fig6

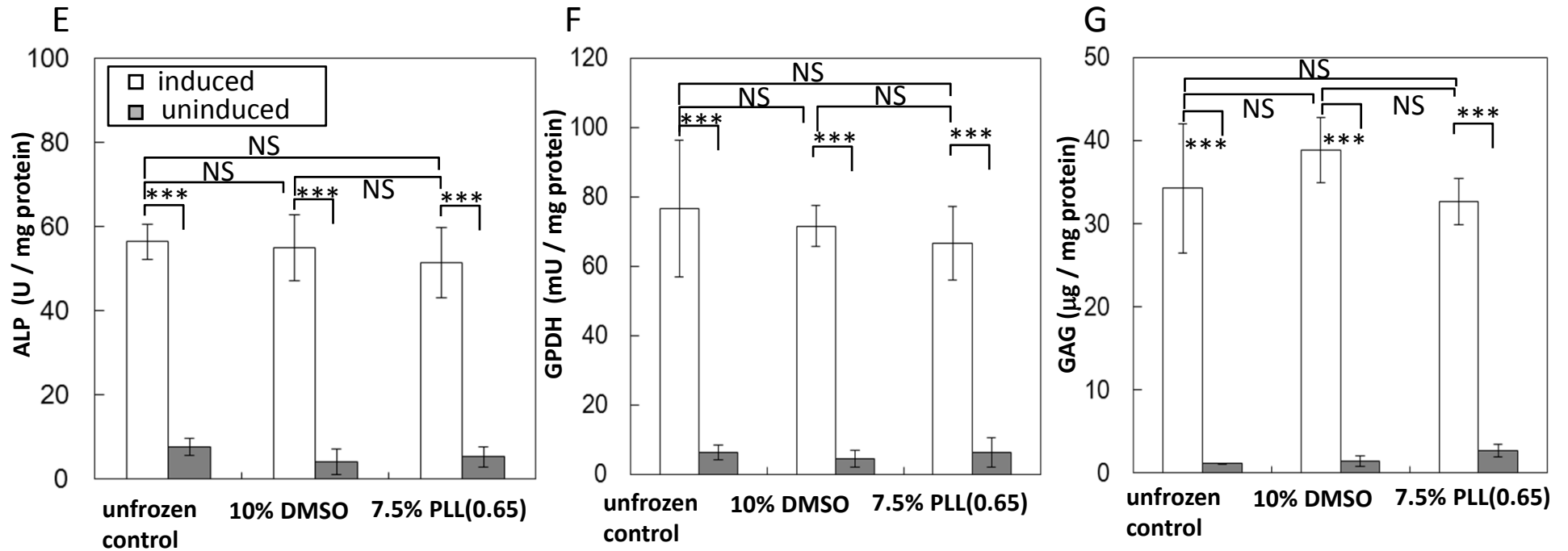
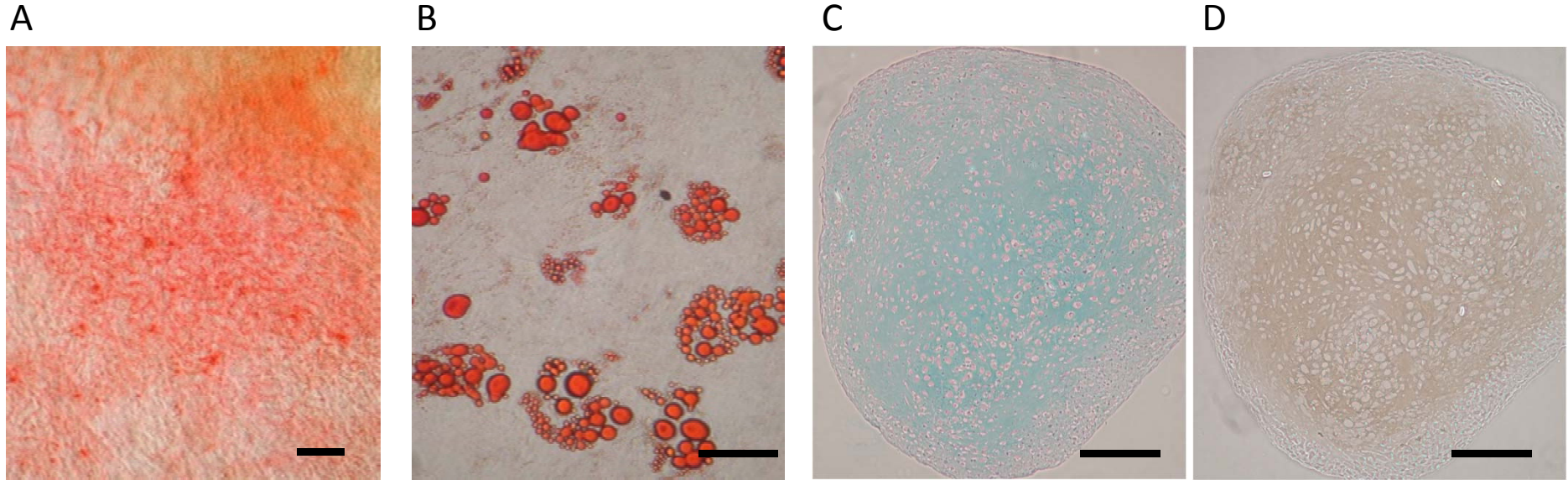


Fig7