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Short Communication

Elimination of contaminants from cell preparations using aqueous two-phase partitioning

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ABSTRACT

Contamination by cell debris and non-viable cells is common in cell-based processes. Cell fragments and dead cells can be produced during culture and in purification steps, and often must be eliminated before analyses, subsequent process steps, and/or final application to avoid interference, fouling and reduced product yields. In the present work, the selective elimination of cell debris from $CD133^+$ stem cells by aqueous two-phase system (ATPS) partitioning is demonstrated. Two conventional ATPS systems showed no selectivity, with 100% of both cell debris and $CD133^+$ stem cells partitioning to the top phase of a Ficoll 400,000-dextran 70,000 system and 100% of both debris and $CD133^+$ cells partitioning to the bottom phase of a PEG 8000-dextran 500,000 system. In a novel UCON–dextran 75,000 system, however, 100% of the $CD133^+$ stem cells partitioned to the bottom phase, while non-viable cells, cell debris and other non-mononuclear cells all partitioned to some extent to the top phase, away from the desired $CD133^+$ cells. $CD133^+$ cell viability was at least 98% after ATPS processing. This result suggests that with larger phase ratios or continuous extraction, this or related ATPS systems could remove essentially all cell debris and non-viable cells with cell yield and viability both near 100%. This approach might find application in a wide variety of cell-based technologies.

1. Introduction

Contaminating cell fragments and dead cells can be produced during culture, particularly under stressful bioreactor conditions, and in downstream process steps, and often must be eliminated before subsequent process steps or final application to avoid handling problems, toxicity to neighbouring cells [1], or reduced product yields. The presence of cell fragments also may interfere with analysis, such as platelet counting [2] or the detection of rare target cells in flow cytometry.

The techniques most commonly used for cell debris removal include centrifugation and filtration in various forms, but these are hindered by the small particle sizes involved, and the small density difference between the contaminants and the cells of interest. Furthermore, not all approaches may be efficiently or quickly implemented at large scale.

Aqueous two-phase systems (ATPS) have been widely exploited for the liquid–liquid extraction of a wide variety of products, including proteins [3–6], cells [7], stem cells [8–11], organelles [12,13], and nucleic acids [14]. These products are separated into immiscible phases by exploiting physicochemical differences among them, including size, density, hydrophobicity, net surface charge, and cell surface properties. ATPS offer several advantages, including biocompatibility even with viable mammalian cells, low cost, scalability, and short processing times. The use of the thermo-precipitable UCON copolymer also allows facile removal and recycling of the polymer by raising the temperature above its cloud point (37 °C) [15,16].

In the present work, the fractionation profile and elimination of cell debris and non-viable cells from $CD133^+$ stem cells by ATPS is demonstrated for the first time, as our previous studies were centered in separating a specific type of stem cells ($CD133^+$) from its most abundant contaminant, red blood cells.

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2. Materials and methods

2.1. Materials

Monoclonal antibodies (mAbs) against human CD34, CD45 and CD133/1 (clone AC133) antigens; human Fc receptor (FcR) blocking reagent; IgGs; and red blood cell lysis solution were obtained from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Dextrans (DEX) produced by *Leuconostoc mesenteroides* with an average molecular weight of 425,000–575,000 g/mol (DEX 500,000) and of 64,000–76,000 g/mol (DEX 70,000 and DEX 75,000), polyethylene glycol (PEG) with a molecular weight of 8000 g/mol (PEG 8000), Ficoll 400,000 g/mol (Ficoll 400,000), Triton X-100, and phosphate buffered saline (PBS) were from Sigma–Aldrich Co. (MO, USA). UCON 50-HB-5100, a thermosensitive 50:50 copolymer of ethylene oxide and propylene oxide (50-HB fluids) with a viscosity of 5100 Saybolt universal seconds at 100 °F (37.8 °C), was purchased from Nufer Plus, S.A. de C.V. (Guanajuato, México). Via-Probe 7-Amino-actinomycin D (7-AAD) was from BD Biosciences (CA, USA), Tris(hydroxymethyl)aminomethane was from Bio-Rad Laboratories, Inc. (CA, USA), sucrose and $MgCl_2$ from DEQ (Monterrey, Mexico), and the density gradient Lymphoprep from Axis-Shield (Oslo, Norway).

The study was conducted with the approval of the Institutional Review Board and Ethical Committee of the Tecnológico de Monterrey School of Medicine and the Hospital San José Tec de Monterrey.

2.2. Human umbilical cord blood (HUCB) donation and cell preparation

HUCB donation and cell preparation were performed as previously reported [15]. Briefly, blood samples were collected by personnel of the Hospital Metropolitano "Dr. Bernardo Sepúlveda" in San Nicolás de los Garza, Mexico, after mothers signed the approved written informed consent form, by puncturing the umbilical cord vein after the cord was cut. The volume of the collected blood varied between 45 and 85 mL and all samples were processed within 18 h after collection. Cell sample preparation consisted of a Lymphoprep density pre-enrichment, red blood cell lysis employing commercial 1X red blood cell lysis solution (Miltenyi Biotec), and a PBS wash, as previously described [15].

2.3. Cell debris sample preparation

The cell debris sample was prepared by partial osmotic lysis of mononuclear cells. To each 10^8 cells (determined using an Invitrogen Countess™ Automated Cell Counter) was added 250 μL of lysis buffer (1.28 M sucrose, 40 mM Tris–HCl pH 7.5, 20 mM $MgCl_2$, and 4% Triton X-100). After ten minutes of incubation, the sample was centrifuged (10 min at 14,000 xg), the supernatant was discarded and the pellet washed with PBS. Afterwards, the sample was centrifuged three minutes (14,000 xg), the PBS decanted, and the required volume of PBS added.

2.4. ATPS construction

Previous work in the group explored different ATPS in order to find the most adequate conditions for stem cells separation. For this, polymer–polymer systems were selected over salt–polymer and the following ATPS were studied, each with the different tie line lengths (15, 20, 25 and 30) and NaCl concentrations (0.01, 0.05, 0.10, and 0.20 M): PEG 4000-DEX 110,000; PEG 10,000-DEX 110,000; PEG 4000-DEX 10,000; PEG 10,000-DEX 10,000; Ficoll 400,000-DEX 70,000; PEG 10,000-Ficoll 400,000; UCON-PEG 8000; UCON–DEX 75,000; and PEG 8000-DEX 500,000. After this preliminary selection, the chosen polymer–polymer systems were PEG 8000-DEX 500,000; Ficoll 400,000-DEX 70,000 and UCON–DEX 75,000 and with these systems different types of solvents were studied (distilled water, PBS, FACS solution), obtaining best results with PBS.

1 g ATPS systems composed of 5.6% PEG 8000–7.5% DEX 500,000, 13.6% Ficoll 400,000–11.6% DEX 70,000 and 6.83% UCON-8.23% DEX 75,000 were constructed in PBS as previously reported [15]. A 100 μL sample (containing on average 1.3×10^7 cells of viable or partially lysed cells) was added to a 1 g ATPS system. All systems were gently mixed at room temperature for 15 min to disperse the phases. Afterwards, the centrifuge tubes containing the dispersed polymers were incubated vertically at 25 °C in a 2 mL tube rack, and complete phase separation was achieved by gravity in less than ten minutes. Visual estimates of the volumes of the top and bottom phases were made using graduated centrifuge tubes. The volumes of the phases were then used to estimate the volume ratio (volume of the top phase/volume of the bottom phase, V_R).

2.5. Sample analysis

A 70 μL sample from the upper and bottom phase of each proposed system was obtained as previously reported [15]. Afterwards, each sample was stained following the manufacturers' recommendations employing FcR reagent, monoclonal antibodies [antiCD34-APC (allophycocyanin), antiCD45-FITC (fluorescein isothiocyanate) and antiCD133/1-PE (phycoerythrin)], and 7-AAD. Flow cytometric analysis of each sample was then performed using a FACSCanto II flow cytometer (BD Biosciences, CA, USA) calibrated daily with 7-Color Setup Beads (BD Biosciences, CA, USA) and analysed by FACSDIVA software (BD Biosciences, CA, USA). Viability and partitioning of $CD133^+$ stem cells and cell debris were determined using a modification of the $CD133^+$ progenitor cell determination by flow cytometry of Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Briefly, the analysis for $CD133^+$ cells was performed excluding cell aggregates, cell debris and platelets in a forward scatter (FSC) versus side scatter (SSC) dot plot (Fig. 1A) followed by gating only the $CD45^+$ cells (Fig. 1B). Afterwards, a 7-AAD versus CD133-PE analysis of all the $CD45^+$ events was performed to include all viable $CD45^+$ cells (Fig. 1C). Next, these gated cells were analysed for expression of CD34 and CD133 markers (Fig. 1D). Finally, the viable $CD45^+$ cells that were also doubly positive for both CD34 and CD133 were analysed to meet all the fluorescence and light scatter criteria of $CD133^+$ stem cells by gating in a FSC versus SSC dot plot (Fig. 1E and F). Dead cells stained with 7-AAD were detected and excluded from the analysis. Additionally, mouse IgG2a-APC and IgG2b-PE staining served as isotype controls. For the cell debris quantification, a special gate from the FSC versus SSC was constructed by comparing the population distributions of samples of viable cells and cell debris. 10,000 events or 1 min acquisition at medium flow rate was used for assessment. The results reported are the average percentages of $CD133^+$ stem cells and cell debris in the top and bottom phase of three experiments ($n = 3$).

3. Results and discussion

The three studied polymer–polymer ATPS were previously selected by our group after a screening of different types of ATPS (polymer–salt and polymer–polymer); solvents (distilled water, PBS, FACS solution); and system parameters [tie line length (TLL) and salt concentration] for the characterization of $CD133^+$ stem cells and red blood cells from whole umbilical cord blood [15]. In