ISOLATING CELLS FROM BLOOD USING BUOYANCY ACTIVATED CELL SORTING (BACS) WITH GLASS MICROBUBBLES

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ABSTRACT

Obtaining pure cell subpopulations is often the first step for a wide range of applications. Here, we present a generic cell isolation strategy, the "buoyancy activated cell sorting (BACS)", using glass microbubbles attached with target-specific antibodies. The design of BACS was centered on the opposite movements of cells and microbubbles during mixing, ultimately improving the capture yield by increasing the number of interactions. Cells labeled with microbubbles will spontaneously float and be isolated without the need for additional separation apparatuses. We demonstrated that more than 99% of CD4 positive cells in whole blood can be isolated in 15 minutes.

KEYWORDS: Buoyancy Activated Cell Sorting (BACS), glass microbubble, cell isolation, particle sorting

INTRODUCTION

The characterization and investigation of specific cell types from a complex mixture of body fluids, such as blood and bone marrow aspirate, requires that the cell of interest can be isolated or purified from other contaminating cells, such as in the applications of cell enumeration [1], cell functional assays [2], and cell-base therapies [3]. Multiple approaches have been employed for sorting cells, ranging from cell physical property-based separation, such as size [4], density [5], and dielectricity [6], to cell biochemical property-based sorting, such as specific cell surface markers [7]. The physical property-based approach is mostly used for pre-processing, while the biochemical property-based approach has been a standard method in obtaining target cells from heterogeneous cell populations. This is conventionally done using fluorescence-activated cell sorting (FACS), magnetic-activated cell sorting (MACS) techniques, and recently microfluidic systems [8]. FACS can obtain highly purified cells, however, the high flow rate required to obtain the high throughput calls for the use of more sensitive and expensive equipment. Hence pre-processes, such as the lysis of red blood cells and the centrifugation are often needed to reduce the load on the system. In contrast to FACS, which interrogates samples on a particle-by-particle basis, MACS involves the mixing of the sample with magnetic beads that have been attached with antibodies or other molecules that recognize the surface marker on the target cell. Bead-bound cells can then be isolated under the influence of a magnetic field. There are also miniaturized FACS and MACS systems that intend to reduce of cost and/or increase the performance of conventional systems [9,10]. Microchannels with immobilized antibodies have also become valuable approaches to sort cells, due to the high surface-area-to-volume ratio and precisely controlled flow conditions [11].

In this work, we developed a novel cell sorting method based on buoyancy activated cell sorting (BACS). A schematic of BACS is shown in Figure 1. Glass microbubbles labeled with target-specific antibodies are added into and mixed with the sample at a fixed rate on a rotatory mixer, causing the microbubbles to float and navigate through the suspension, which provides a fertile means for the contact of microbubbles and target cells. Target cells attached with glass microbubbles will then float and be separated spontaneously by the augmented buoyancy force. Microbubbles are widely used for ultrasound contrast imaging in clinics. Perfluorocarbon gas-filled microbubbles with modified surface properties have been shown to specifically bind to red blood cells *in vitro* [12]. In contrast, our method uses commercially available glass microbubbles that are inexpensive, more stable, and which size can be tightly controlled and surface can be modified using various protocols developed for glass substrate. We have also demonstrated that anti-human CD4 antibody modified glass microbubbles can selectively bind to CD4 positive cells in whole blood with high efficiency. Finally, we investigated the dependence of sorting efficiency on the mixing time.

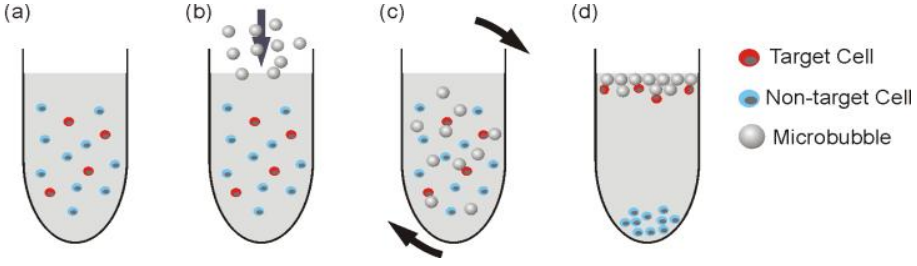

Figure 1: A schematic of buoyancy activated cell sorting (BACS). Surface-functionalized glass microbubbles bind to target cells after a brief rotary mixing (a-c). Cells attached by glass microbubbles float and are separated spontaneously by buoyancy (d).

EXPERIMENTAL

Surface modification of glass microbubbles. In this study, we used glass microbubbles iM30K from 3M™ (St. Paul, MN). These are hollow glass microspheres of high-strength. The average diameter and density of the glass microbubbles is 18 μm and 0.6 g/cm^3, respectively. Glass microbubbles were functionalized with anti-CD4 antibody for capturing target CD4+ T cells. The immobilization of antibody on the glass was achieved using a previously described method based on avidin-biotin chemistry [13,14]. Briefly, the glass microbubbles were pretreated with 1:1 (v/v) methanol (HPLC grade, Fisher Scientific, Pittsburgh, PA) / HCl (Fluka Chemie AG, Ronkonkoma, NY) for 30 minutes followed by a bath in concentrated H_2SO_4 (96%, CMOS grade, Mallinckrodt Baker, Phillipsburg, NJ) for 30 minutes. The glass microbubbles were then exhaustively rinsed in deionized water, dried under a stream of nitrogen, and treated with 4% (v/v) solution of 3-mercaptopropyl trimethoxysilane (Gelest, Morrisville, PA) in ethanol (200 proof, Fisher Scientific, Fair Lawn, NJ) for 60 min at room temperature, followed by an incubation of 0.01 μmol/mL N-[γ-maleimidobutyryloxy]succinimide ester (Pierce Biotechnology, Rockford, IL) in ethanol for 30 min at room temperature. Next, the glass microbubbles were incubated with 10 μg/mL NeutrAvidin (Pierce Biotechnology, Rockford, IL) solution in PBS (Mediatech, Herndon, VA) for 1 h at 4 ºC. Finally, 10 μg/mL biotinylated anti-CD4 antibody (clone 13b8.2, Beckman Coulter, Somerset, NJ) solution in PBS containing 1% (w/v) BSA (Sigma Aldrich, St. Louis, MO) and 0.09% (w/v) sodium azide (Sigma Aldrich, St. Louis, MO) was added and allow to react with NeutrAvidin at room temperature for 15 min to complete the antibody immobilization. Glass microbubble containing solutions were rocked on a rotator mixer during each incubation step. After each step, the surfaces were rinsed with either ethanol or PBS, depending on the solvent used in the previous step, to flush away unreacted molecules. Glass microbubbles were rinsed with PBS containing 1% (w/v) BSA before use.

Collection of blood samples. Blood samples from healthy subjects were obtained through the Massachusetts General Hospital in Boston, MA under the institutional review board (IRB) approved protocols. Samples of 5 mL of peripheral blood were collected by venipuncture into Vacutainer collection tubes containing the anticoagulant K_2EDTA (BD Biosciences, Franklin Lakes, NJ). All samples were run on BACS on the day of blood collection.

Buoyancy activated cell sorting experiments. 10 μL whole blood samples were mixed with 50 μL of antibody modified glass microbubbles of concentration 10^6 microbubbles/mL at 6 rpm on a rotatory mixer (Mix-All™ Laboratory Tube Mixer, RPI, Mt. Prospect, IL) for various lengths of time (1~30 min).

Flow analysis. Alexa Fluor® 488-conjugated mouse anti-human CD4 (clone RPA-T4), Alexa Fluor® 647-conjugated mouse anti-human CD3 (clone UCHT1), and phycoerythrin (PE)-conjugated mouse anti-human CD14 (clone M5E2) were obtained from BD Bioscience (San Diego, CA). In order to confirm the efficiency of the glass beads in depleting target cells from whole blood, samples before and after BACS were collected and treated with ammonium chloride lying solution for 5 min to lyse erythrocytes. Next, samples were washed with PBS containing 1% BSA (w/v) and stained with an antibody mixture containing AF647-anti-CD3/AF488-anti-CD4/PE-anti-CD14 for 15 min. After rinsing off the unbound antibody with PBS, samples were analyzed using standard flow cytometry to quantify the percentage of CD4+ T cells. The flow cytometric measurements were performed on a FACSCalibur (Beckton Dickinson Immunocytometry System (BDIS), San Jose, CA) instrument using BD CellQuest Pro Software. The capture efficiency, or yield of BACS was estimated from the ratio of the percentage of CD3+ CD4+ T cells in samples collected before and after BACS.

RESULTS AND DISCUSSION

We have developed a novel strategy for the isolation and enrichment of cell subtypes from biologically complex fluids, such as peripheral whole blood. We demonstrated that anti-human CD4 antibody modified glass microbubbles can selectively bind to CD4 positive cells in whole blood as shown in Figure 2.

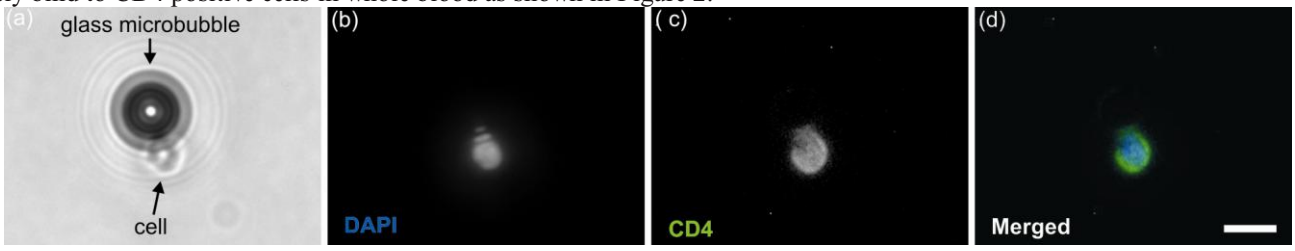

Figure 2: Micrographs of a CD4 T cell sorted from whole blood using BACS and stained with DAPI and anti-CD4 antibodies. (a) A phase-contrast image. (b-d) Merged images (scale bar = 10 μm).

The dependence of sorting efficiency on the mixing time was evaluated by flow cytometry and is shown in Figure 3. We observed that more than 90% of the target cells can be isolated from whole blood literally after only 6 flips of the mixture and the yield increases to above 95% when the mixing time ranges from 5 to 30 minutes.

CONCLUSION

In conclusion, we invent and demonstrate a simple, quick and inexpensive cell sorting method based on glass microbubble cell affinity buoyancy. Minimum handling procedures, rapid operation, and a high performance makes this strategy a versatile method that could be applied to a number of applications where specific and efficient cell isolation is required, and could be potentially applied in resource limited settings.

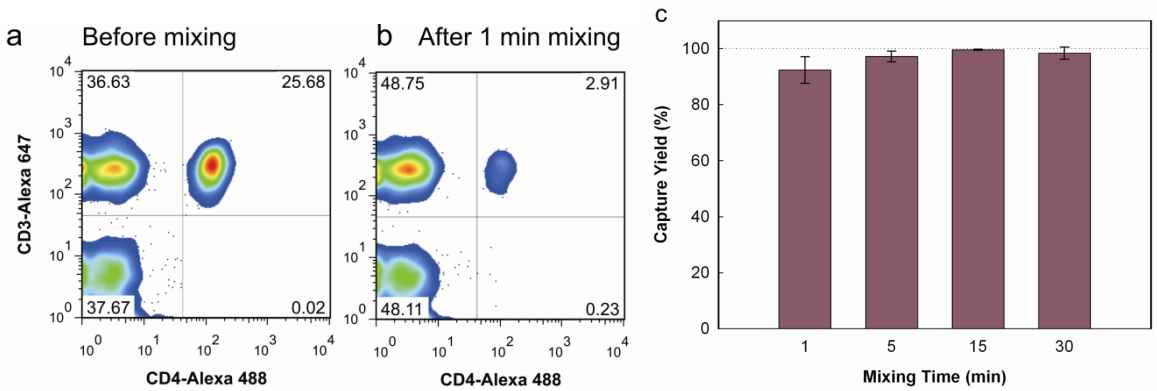

Figure 3: Dependence of capture yield on the mixing time in BACS evaluated by flow cytometry using 10 μL blood samples from healthy subjects. (a) Flow cytometric analysis of a blood sample before CD4+ T cell isolation. Cells were acquired in the gated lymphocyte population, and the quadrants were set up with an isotype-matched control. The CD4+ T cells (CD3+CD4+) compose 25.68% of all lymphocytes. (b) Flow cytometric analysis of the same blood sample after CD4+ T cell depletion using BACS. 10 μL of whole blood were mixed with 50 μL of glass microbubble suspension for 1 minute on a mixer rotated at 6 rpm. The composition of the target cells in the sample dropped to 2.91% of all lymphocyte population. (c) Capture yield with different mixing times calculated from flow cytometric analysis. More than 90% of the target cells can be isolated from whole blood after 1 minute of mixing with glass microbubbles. The yield increases to above 95% with the mixing time ranging from 5 to 30 minutes. Each data point was repeated in triplicates using different blood samples. The error bars represent standard deviations.

ACKNOWLEDGEMENTS

This work was supported by NIH-P41EB002503. The authors thank Octavio Hurtado for microfabrication training and expert technical support.

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