Fast sorting of CD4+ T cells from whole blood using glass microbubbles

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The isolation of CD4 positive T lymphocyte (CD4+) from peripheral blood is important for monitoring patients after HIV infection. Here, we demonstrate a fast isolation strategy for CD4+ cells that involves mixing blood and glass microbubbles. After the specific binding of target cells to the microbubbles carrying specific antibodies on their surface, target cells will spontaneously float to the top of the blood vial and can be quickly separated. Using this strategy, we demonstrate that the isolation of CD4+ cells in less than 5 minutes and with better than 90% efficiency. This strategy for cell isolation based on buoyancy and glass microbubbles is quick and inexpensive, minimizes blood handling, does not require magnetic fields, or centrifugation equipment, and could lead to new, efficient strategies for AIDS diagnosis in resource-limited areas.

INNOVATION

Separating CD4 positive T lymphocytes from peripheral blood is an important step in enumerating CD4+ T cells for monitoring AIDS. Despite numerous devices have been developed, low-cost medical devices for improving patient care and treatment outcome in developing countries are still needed. Our specific innovation is the strategy of using antibody-modified glass microbubbles to perform affinity-based CD4+ T cell sorting with a simple setup; this may further reduce the cost and simplify the procedure of AIDS monitoring for resource limited settings.

INTRODUCTION

Separating pure cell subpopulations from the mixture of cells in blood is often the first step of a wide spectrum of research and clinical applications, including cell enumeration¹, cell functional assays², and cell-based therapies³. For human immunodeficiency virus (HIV) infected patients the number of CD4+ T lymphocytes in peripheral blood is an important marker for monitoring disease progression to AIDS and treatment efficacy⁴. The standard method for enumerating CD4+ T cells is by using fluorescence-activated cell sorting (FACS) technique⁵. While FACS machines are commonly accessible in developed countries, its high equipment and operation costs have limited their uses in resource-limited areas where most of the HIV-infected subjects reside. On the other hand, magnetic-activated cell sorting (MACS) technique has also been utilized for CD4 T cell counting^{6,7} as an attempt to bring down the initial equipment cost to make CD4 T cell testing more affordable. 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. Although MACS has largely reduced the initial equipment

cost, it remains relatively expensive at ~ 12 – 22 USD per test⁸ and its utility is limited for separating CD4+ T lymphocyte in resource limited areas.

In this work, we proposed an alternative strategy for CD4+ T lymphocyte separation from whole blood that uses glass microbubbles and buoyancy for separation. We termed this method “buoyancy-activated cell sorting (BACS)” (Fig. 1). Specifically, we labeled glass microbubbles with target-specific antibodies and mixed the microbubbles with blood samples in a tube. Flipping the tube a few times causes the microbubbles to float and navigate through the suspension, and provides a simple and efficient means for enhancing the contact between microbubbles and target cells. After mixing, target cells attached with glass microbubbles are lifted by the augmented buoyancy force, while the non-target cells sediment to the bottom of the tube due to the gravity.

METHODS AND MATERIALS

Glass microbubbles

We used glass microbubbles iM30K from 3MTM (St. Paul, MN). These are hollow glass microspheres of high-strength, typically used for a variety of coating formulations. The average diameter of the glass microbubbles is 18 μm and the density is 0.6 g/cm³ (see **Supplementary Fig. 1**).

Surface modification of glass microbubbles

Glass microbubbles were functionalized with anti-CD4 antibody for capturing target CD4+ T cells. The immobilization of antibody on the glass was achieved using avidin-biotin chemistry as described elsewhere^{9–11}. 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 H₂SO₄ (96%, CMOS grade, Mallinckrodt Baker, Phillipsburg, NJ) for 30 minutes. The glass microbubbles were then

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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).

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 minutes at room temperature, followed by an incubation of 0.01 $\mu\text{mol/mL}$ N-[γ -maleimidobutyryloxy]succinimide ester (Pierce Biotechnology, Rockford, IL) in ethanol for 30 minutes at room temperature. Next, the glass microbubbles were incubated with 10 $\mu\text{g/mL}$ NeutrAvidin (Pierce Biotechnology, Rockford, IL) solution in PBS (Mediatech, Herndon, VA) for 1 hour at 4 $^{\circ}\text{C}$. Finally, 10 $\mu\text{g/mL}$ 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 minutes 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

Ten microliters of whole blood samples were mixed with 50 μL of antibody modified glass microbubbles of concentration 5×10^6 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 minutes).

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 lysis solution for 5 minutes 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 minutes. 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.

Glass microbubble staining

The NeutrAvidin coated glass microbubbles were incubated with PE-conjugated biotin (Invitrogen, Camarillo, CA) for 15 minutes. The microbubbles were then rinsed with excess PBS to remove unbound PE-conjugated biotin molecules in the solution.

Cell staining

To image cells captured on the glass microbubble from whole blood using BACS, the top portion of the sample after BACS containing glass microbubbles and captured cells were rinse with PBS and fixed with 1% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS solution for 20 minutes, incubated with Alexa Fluor® 647-conjugated mouse anti-human CD3 antibody (BD Biosciences, San Jose, CA) for 15 minutes, followed by 0.1% DAPI (Sigma Aldrich, St. Louis, MO) and 0.2% Triton-X 100 (Sigma Aldrich, St. Louis, MO) in PBS solution for 30 minutes, followed by being imaged on an inverted microscope (Nikon Eclipse TE2000, Nikon Inc., Tokyo, Japan).

Theoretical background

Two major aspects affecting the capture efficiency in this system are the volume searched per glass microbubble and the detaching force exerted on the cell-microbubble complex. The larger volume searched per glass microbubble per unit time, the greater the possibility for the glass microbubble to encounter a target cell, and hence the better sorting efficiency. A fast traveling microbubble should search more sample space, however, it also experiences more friction force exerted by its surrounding fluid. Therefore, the maximum velocity of the microbubble should be kept smaller than the speed where the action-reaction force between the microbubble and the bound target cell equals the rupture force between the antigen on the target cell and the antibody conjugated on the glass microbubble in this system. Both aspects are discussed below.

In order for any cells to come into contact with the microbubble, they must lie along streamlines that bring them to the distance less than the radius of the cell from the surface of the microbubble. Raising microbubbles reach a steady speed depending on their radius, density and the physical properties of the fluid they are traveling through (Equation 1).

$$v_{\theta}(r, \theta) = U \sin \theta \left(1 - \frac{3}{4} \frac{R}{r} - \frac{1}{4} \left(\frac{R}{r} \right)^3 \right). \quad (1)$$

The radii of microbubble and cell are denoted R_b and R_c respectively. By integrating Equation (1) through the annulus $R_b < r < R_b + R_c$ at the equatorial plane allows us to obtain the volume of fluid searched by one microbubble per unit time, Q .

$$Q = \pi U_b R_c^2 \left[1 + \frac{1}{2 \left(1 + \frac{R_c}{R_b} \right)} \right]. \quad (2)$$

Figure 2 Steady flow of a viscous fluid at very low Reynolds numbers (“creeping flow”) past a glass microbubble. (a) The flow lines are shown in a planar section parallel to the flow direction and passing through the center of the microbubble. Coordinates for description of the theoretical distribution of velocity in flow past a microbubble are also shown. (b) The force diagram of a glass microbubble-cell complex with the internal action-reaction force also shown. (c) A plot shows the dependence of the volume of fluid searched per microbubble per unit time, Q (the solid line), as well as Q_n , Q normalized to the cross-sectional area per microbubble (the dashed line), to the diameter of the microbubble. (d) A plot shows the dependence of the maximum shear stress (the dashed line) and the action-reaction force, F_{act} (the solid line), to the diameter of the microbubble.

Thus, assuming each cell-microbubble collision results in capture, the maximum number of cells that could be collected per unit time can be calculated by multiplying Equation (2) by the concentration of the cell and that of the glass microbubbles. The dependence of Q and Q_n to the diameter of the microbubble is plotted in Fig. 2c, while R_c is fixed to the average radius of target lymphocytes, $4 \mu\text{m}$. Q_n is Q normalized to the cross-sectional area of the microbubble, representing a real volume searched per cross-sectional area. As indicated in Equation (2), Q is proportional to U_b and R_c^2 when $R_b \gg R_c$ (the solid line). However, Q_n only increases very modestly with the size of the microbubble (the dashed line) and reaches to 90% of its maximum value when R_b equals to $2.3R_c$.

Cell-microbubble interaction forces

The force diagram of a microbubble-cell complex is shown in Fig. 2b. When a non-neutrally buoyant body is released from rest in a still fluid, it accelerates in response to the force of gravity and buoyancy. As the velocity of the body increases, the oppositely directed drag force, F_d , exerted by the fluid grows until it eventually equals the submerged weight of the body, whereupon the body no longer accelerates but falls (or rises) at its terminal velocity. At every point on the surface of the microbubble there

is a definite value of fluid pressure (normal force per unit area) and of viscous shear stress (tangential force per unit area). These values also can be obtained analytically from Stokes’ solution for creeping flow around the microbubble. Both maximum values are found to be the same and equal to $3\mu U_b/2R_b$. Details about the governing equations can be found in the **Supplementary Information**. Note that we include the internal action-reaction force, F_{act} , between the microbubble-cell complex, which should be smaller than the rupture force between the antigen on the target cell and the antibody conjugated on the glass microbubble for the stable microbubble-cell complex formation. The dependence of maximum shear stress and to the diameter of the microbubble is shown in Fig. 2d. The maximum shear stress is linearly proportional to the diameter of the microbubble and its value is orders of magnitude smaller than F_{act} , when integrated over the cross-sectional area of the cell, indicating the friction drag force exerted by the fluid on the bound cell is much smaller than the interaction force between the microbubble-cell complex.

RESULTS AND DISCUSSION

We have developed a strategy for fast separation of cells from whole blood in resource limited setting. After mixing microbubbles with whole blood,

Figure 3 Images of glass microbubbles labeled with PE-conjugated biotin. Unmodified glass microbubbles (as shown in the phase-contrast image **a**) are not labeled with PE-conjugated biotin as there is no fluorescence shown in the fluorescence image **b**, whereas the surface-modified glass microbubbles (as shown in the phase-contrast image **c**) have bright ring-shaped fluorescence shown in image **d**, indicating that the PE-conjugated biotin molecules are immobilized on the surface of glass microbubbles by their NeutrAvidin coating (scale bar = 50 μm).

the movements in opposite directions of cells (pulled down by gravity) and glass microbubbles (lifted by buoyancy) facilitates the capture of target cells by significantly increasing the interactions of target cells with the antibody-coated glass microbubbles. Successful immobilization of NeutrAvidin on glass microbubbles was verified by the specific binding of PE-conjugated biotin to the NeutrAvidin on the microbubbles surface (**Fig. 3**). After incubation with biotinylated anti-human CD4 antibody, the modified glass microbubbles can selectively bind to CD4 positive cells in whole blood (**Fig. 4**).

The dependence of sorting efficiency on the mixing time was evaluated by flow cytometry (**Fig. 5**). After one minute the blood sample and microbubbles in a vial (the equivalent of only 6 flips of the vial), we observed that 90% of the target cells can be isolated from whole blood. Increasing the mixing time to 30 minutes, increases the yield to above 95%. MACS-based CD4⁺ T cell isolation has been previously reported to have the yield of ~86%–93%¹² and ~91%–94%¹³ using blood and thymus effluent respectively. The high yield of the method compared to methods employing magnetic beads, could be explained by the enhanced interactions between the glass microbubbles and the blood cells. We estimate using Equation (2) that the total volume searched by the number of glass microbubbles per minute was 42 μL , which is in a good agreement of results shown in **Fig. 2c**. Although the sorting efficiency can potentially be further increased by increasing the microbubble size, and consequently increasing the “volume searched per microbubble”, the action-reaction force will also increase,

which could detach the bonded cells from the glass microbubbles. The forces required to rupture various single antibody-antigen complexes have been probed by using atomic force microscopy and found to be in the range of 35 ~ 1029 pN^{14–16}, while the interaction force between NeutrAvidin and biotin has been probed to be 165 pN¹⁷. Our theoretical calculations indicate that for microbubbles with diameter below 25 μm the force between cells and the surface is less than 35 pN by Equation (5), and thus below such threshold.

As the importance of global health has become increasingly recognized, low-cost medical devices are needed for improving patient care and treatment outcome in developing countries for infectious diseases such as AIDS. This has led to the development of miniaturized FACS and MACS systems that intend to reduce of cost and/or increase the performance of conventional systems^{18,19}, and microchip-based method that used microchannels with immobilized antibodies to sort cells²⁰. One critical advantage for microbubbles is their reduced cost, considering that they 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*²¹. They have also been demonstrated in isolating tumor cells²². However, due to the instability of Perfluorocarbon gas-filled microbubbles in whole blood, only plasma-depleted blood samples could be used²². In contrast, our method uses commercially available glass microbubbles that are inexpensive, more stable, and the surface can be modified using various protocols developed for glass substrate. We have also demonstrated that

Figure 4 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 of a glass microbubble attached to a cell. (b–d) Merged fluorescence images identify a CD4⁺ T cell (scale bar = 10 μ m).

anti-human CD4 antibody modified glass microbubbles can selectively bind to CD4 positive cells in whole blood with high efficiency. The target cells may be washed and eluted by inserting a pipette tip into the tube to add and aspirate the buffer/solution underneath the floating microbubbles. In conclusion, we developed a new cell sorting method for CD4⁺ T lymphocyte isolation from whole blood using glass microbubbles and buoyancy. The method minimizes sample handling, is fast, requires no expensive equipment, and could be an attractive alternative to existing methods for monitoring CD4⁺ T lymphocyte cell counts in resource limited settings.

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Figure 5 Dependence of capture yield on the mixing time in BACS evaluated by flow cytometry using 10 μ L 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. Ten microliters of whole blood samples were mixed with 50 μ L 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.

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Table 1 Glass microbubble and CD4+ T lymphocyte characteristics.

	Diameter (μm)	Density (g/cm^3)	Terminal velocity ($\mu\text{m}/\text{s}$)	Re
Glass microbubble	14 ± 3	0.6	42.6 (rising)	0.0003
CD4+ T lymphocyte	7–8	1.06	2.1 (settling)	0.000008

SUPPLEMENTARY INFORMATION

S1. Characterization of glass microbubbles and surface modification

Glass microbubbles were imaged on an inverted microscope (Nikon Eclipse TE2000, Nikon Inc., Tokyo, Japan). The diameters of the microbubbles were analyzed using an image processing software (ImageJ, National Institutes of Health). Briefly, images were converted to binary images by manually setting light intensity thresholds after subtracting the background. Diameters were calculated from the area assuming circular geometry. The average diameter was $14 \pm 3 \mu\text{m}$. Total 3406 microbubbles were analyzed.

Supplementary Figure 1 The size distributions of glass microbubbles.

S2. Theory and modeling

S2.1 Searched volume

Consider the cell can only become captured if the fluid streamline brings the cell into direct contact with the collecting surface of the glass microbubble and the center of the cell follows the streamline, an assumption that is reasonable for this system when the Reynolds number is sufficiently small. The Reynolds numbers for the rising glass microbubble (Re_b) and the settling cells (Re_c) can be calculated using Equation (1) and are all < 1 using the values shown in Table 1.

$$Re_b = \frac{\rho U_b D_b}{\mu} \quad Re_c = \frac{\rho U_c D_c}{\mu} \quad (\text{a})$$

where ρ is the density and μ is the viscosity of the fluid, U_b , U_c and D_b , D_c are the terminal velocities and diameters of the microbubble and the target cell, respectively. The terminal velocities U_b and U_c can be calculated by

$$U_b = \frac{2R_b^2(\rho - \rho_b)g}{9\mu} \quad U_c = \frac{2R_c^2(\rho_c - \rho)g}{9\mu} \quad (\text{b})$$

where ρ_b and ρ_c are the densities of the microbubble and the cell, respectively. At very low Reynolds numbers, $Re \ll 1$, an approaching flow is so slow that accelerations of the fluid as it passes around the microbubble can be ignored, resulting in an equation that can be solved analytically following Stokes' law. Equations (1) and (1.1) give the theoretical distribution of velocity v , as a function of distance r from the center of the microbubble and the angle θ measured around the microbubble from 0° at the front point to 180° at the rear point as shown in Fig. 2a. The velocity of the fluid is everywhere zero at the microbubble surface and increases only slowly away from the microbubble, even in the vicinity of the equatorial plane. The flow lines are deflected as they pass around the microbubble, even streamlines infinitely far away are displaced to satisfy continuity condition as $v \leq U$ everywhere near the equatorial plane.

$$v_r(r, \theta) = U \cos \theta \left(1 - \frac{3R}{2r} + \frac{1}{2} \left(\frac{R}{r} \right)^3 \right) \quad (1)$$

$$v_\theta(r, \theta) = U \sin \theta \left(1 - \frac{3R}{4r} - \frac{1}{4} \left(\frac{R}{r} \right)^3 \right). \quad (1.1)$$

By integrating Equation (1.1) through the annulus $R_b < r < R_b + R_c$ at the equatorial plane allows us to obtain the volume of fluid searched by one microbubble per unit time, Q .

$$Q = \pi U_b R_c^2 \left[1 + \frac{1}{2 \left(1 + \frac{R_c}{R_b} \right)} \right] \quad (2)$$

S2.2 Cell-microbubble interaction forces

At every point on the surface of the microbubble there is a definite value of fluid pressure (normal force per unit area) and of viscous shear stress (tangential force per unit area). These values also can be obtained analytically from Stokes' solution for creeping flow around the microbubble using Equations (1) and (1.1) and can be expressed in Equations (3) and (4), respectively.

$$\tau_{r\theta} = \frac{3}{2} \frac{\mu U_b}{R_b} \left(\frac{R_b}{r} \right)^4 \sin \theta \quad (3)$$

$$P = P_0 - \rho g z - \frac{3}{2} \frac{\mu U_b}{R_b} \left(\frac{R_b}{r} \right)^2 \cos \theta \quad (4)$$

where P_0 is the free-stream pressure. The maximum shear stress happens around the midsection when $\sin \theta$ equals to 1, while the pressure is high in the front and low in the back of the microbubble when $\cos \theta$ equals to 1 and -1 , respectively. Both maximum values are found to be the same and equal to $3\mu U_b/2R_b$, when the height of the microbubble inside the fluid, z , is ignored.

The internal action-reaction force, F_{act} , between the microbubble-cell complex, which should be smaller than the rupture force between the antigen on the target cell and the antibody conjugated on the glass microbubble for the stable microbubble-cell complex formation. F_{act} can be expressed as

$$F_{act} = \frac{4\pi g R_c R_b}{3(R_c + R_b)} \left[R_c^2(\rho_c - \rho) + R_b^2(\rho - \rho_b) \right]. \quad (5)$$