Microfluidic aqueous two phase system for leukocyte concentration from whole blood

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Abstract Leukocytes from a whole blood sample were concentrated using a microfluidic aqueous two phase system (μ ATPS). Whole blood was simultaneously exposed to polyethylene glycol (PEG) and dextran (Dex) phase streams and cells were partitioned based on their differential affinity for the streams. The laminar flow characteristic of microfluidic devices was used to create zero, one, and two stable interfaces between the polymer streams. Three different patterns of three polymer streams each were evaluated for their effectiveness in concentrating leukocytes: immiscible PEG-PEG-Dex, immiscible Dex-PEG-Dex, and miscible PEG-PBS-Dex. The most effective configuration was the Dex-PEG-Dex stream pattern which on average increased the ratio of leukocytes to erythrocytes by a factor of 9.13 over unconcentrated blood.

Keywords Microfluidics · Leukocyte · Aqueous two phase separation · Blood · PEG · Dex

1 Introduction

An emerging theme in microfluidics is the use of miniaturized devices for acquiring cellular-level diagnostic information from patient whole blood samples (Lauks 1998). The ability to concentrate leukocytes from whole

blood is a prerequisite for most hematological analyses. A sample of whole blood contains $\sim 5 \times 10^6$ erythrocytes/ μl which, if not removed, can interfere with assays aimed at the less common leukocytes ($\sim 10^4/\mu\text{l}$). Within the context of clinical diagnostics, leukocyte concentration from whole blood is typically performed by lysing the erythrocytes and separating the leukocytes from the debris by centrifugation. This method requires milliliters of sample, is time- and labor-intensive, and requires the use of trained personnel and specialized equipment. Furthermore, lysing solutions can permeabilize the surface of leukocytes (Tiirikainen 1995) and, within twenty minutes, lyse them. Lysing reagents have also been shown to affect the expression of CD adhesion glycoproteins on the leukocyte surface. These surface markers typically used for characterizing the remaining cells via flow cytometry (Lundahl et al. 1995). Thus, next-generation point-of-care devices that perform leukocyte analysis will require alternative methods of partitioning blood that use smaller sample volumes and minimize damage to leukocytes.

Separation of blood cells in microfluidic devices has recently become an active area of research. Many different separation methods have been demonstrated such as lateral displacement (Huang et al. 2004), filters (Carlson et al. 1997), lysis (Sethu et al. 2004), and hydrodynamic separation (Yamada et al. 2004a). For an overview of microfluidic blood separation methods see the review by Toner et al. (Toner and Irimia 2005). While each method is capable of isolating cells, they also have disadvantages which must be weighed for the envisioned application. Of these methods only lysis has a specificity near 100% and even with its drawbacks

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it still remains the method of choice for leukocyte concentration.

One microfluidic separation method which has yet to be applied to blood is an aqueous two-phase system (ATPS). ATPS have been demonstrated in microfluidic devices for separating plant cell aggregates (Yamada et al. 2004b), live/dead Chinese Hamster Ovary cells with fractionation efficiencies of up to 97% (Nam et al. 2005), and for protein purification (Meagher et al. 2008). However, no work has been done using ATPS to microfluidically separate leukocytes from erythrocytes, even though these cells types are known to have different surface properties, which suggests ATPS should be a viable separation strategy.

An ATPS is formed by mixing two immiscible polymers together in solution. Commonly used polymers for macroscale ATPS separations are 4% w/w polyethylene glycol (PEG) and 5% w/w dextran (Dex) (Albertsson 1986). The original work on using ATPS for cell separations was performed 50 years ago by Per-Ake Albertsson in which he demonstrated partitioning of bacteria and cell fragments (Albertsson 1956, 1986). Since then ATPS has been shown to be effective at separating erythrocytes based on age (Walter and Krob 1989) and species (Walter et al. 1976). ATPS has also been used to analyze the surface charge properties of blood cells (Pinilla et al. 1994; Walter et al. 1980a) and to fractionate leukocyte subtypes (Walter et al. 1979, 1980b; Michalski et al. 1986; Malstrom et al. 1986; Levy et al. 1981). Leukocyte fractionation has traditionally been done using a multi-stage ATPS approach called counter current distribution (CCD) that can improve results.

Separation of blood components with ATPS at the macroscale requires large sample volumes and can take 20 minutes to complete. Often multiple separations are required to achieve well-sorted samples. Microfluidic devices can potentially enhance ATPS because the surface area-to-volume ratio of the streams is very large, decreasing the distance a cell must travel before coming into contact with a phase interface and potentially making separations faster. Microfluidics also allows for continuous cell separation, something that is difficult with traditional ATPS.

2 Theory

Each cell type has particular surface properties and net charge; within an ATPS each phase has a unique surface energy and charge. When a heterogeneous mixture of cells is placed in an ATPS, the distinctive characteristics of each cell determine its interaction with

the phases, resulting in cell separation by type. The cells will position themselves in the most energetically favorable location within the system, which can be in either phase or at the interface between the phases. The forces acting on a cell within an ATPS result from electrostatic potential and surface energy (Walter et al. 1982; Gerson 1980) and are shown in Fig. 1.

PEG and Dex are non-ionic, so salts must be added to give the ATPS a physiological osmolarity. Salts can also be used to adjust the electrostatic potential between the phases because certain ions partition unevenly within each polymer phase. For example, while sodium and chloride ions distribute evenly between PEG and Dex phases resulting in no net charge, phosphate ions separate unevenly making PEG positive relative to Dex. If the generated electrostatic potential is large enough, it will interact with the surface charge of cells, driving them into the phase of opposite charge. Typical electrostatic potentials range from microvolts to millivolts and usually have less of an influence on cell separation than the surface energy. However, even relatively weak electrostatic potentials can generate strong electric fields over the short distances present in microfluidic systems, making this force potentially useful for cell sorting applications.

The other force present in ATPS, surface energy, is strong enough to drive cells into either phase or hold them at the interface. If cells experience a differential surface energy between the bulk PEG and Dex phases

Fig. 1 A cell at a two-phase interface experiences forces from the electrostatic potential (EP) and surface energy (SE) of the phases. Phosphate ions separate unevenly between the two-phases generating a positive potential in PEG relative to Dex. Differential surface energies $\gamma_{Cell-Dex}$, $\gamma_{Cell-PEG}$, and $\gamma_{PEG-Dex}$ between the phases and the cell will determine its final position. Surface energy differences cause blood cells to move into the Dex phase within the PEG-Dex system

that is greater than the PEG-Dex interface surface energy, or

$$|\gamma_{(\text{Cell-PEG})} - \gamma_{(\text{Cell-Dex})}| > \gamma_{(\text{PEG-Dex})} \quad (1)$$

then they will move into the phase in which they experience the lowest surface energy. Otherwise the cells will be held in place at the interface.

The magnitude of force experienced by a cell from ATPS surface energy is proportional to the cell surface area, surface properties and polymer concentration. By minimizing the PEG and Dex polymer concentrations, the difference between the surface areas of erythrocytes and leukocytes ($\sim 140 \mu\text{m}^2$ and $\sim 300 \mu\text{m}^2$, respectively) can be used to concentrate the leukocytes. The erythrocytes have a lower surface energy and will quickly move into the Dex phase while the leukocytes, with a higher surface energy, will stay near the interface.

Microfluidics provides an advantage here because the large surface-to-volume ratio allows many cells to rapidly sample the interface and thus determine their placement either within the bulk phases or at the interface. The high viscosity of the phases and the microscale dimensions of the system ensure that $\text{Re} \ll 1$ which will prevent turbulence and allow smooth, continuous interfaces to be maintained. Furthermore, the laminar flow present in microfluidic channels allows cells to be precisely positioned near the interface, further enhancing cell sampling of the two-phases.

3 Device design and fabrication

Microfluidic devices were fabricated using the techniques of soft lithography and polydimethylsiloxane (PDMS) replica molding (Whitesides et al. 2001). Briefly, micromolding masters were created by spin coating negative tone photoresist to a height of $100 \mu\text{m}$ (SU-8 2100, Microchem Corp., Newton MA) on a silicon wafer. The photoresist was selectively exposed to ultraviolet light (BlakRay B-100a, UVP, Upland, CA) through a high resolution transparency containing the channel design. After the unexposed photoresist was removed via developer, a PDMS mold was cast off the master as follows (SYLGARD 184 Silicone Elastomer Kit, Dow Corning, Midland, MI). PDMS base and curing agent were mixed in a 10:1 ratio, degassed, and poured over the mold which was held in an aluminum foil boat. The PDMS was then cured at 125°C for 15 min. Once the PDMS was removed from the master, ports were cored out and the PDMS was bonded to a glass slide using an oxygen plasma treatment. Tygon tubing (Tygon S-50-HL, Cole-Palmer, Vernon Hills, IL)

was inserted into ports, cored out with a blunt 16 gauge needle, and used to connect the microfluidic devices to syringe pumps.

The ATPS microdevices had three inputs which merged into a single main channel and then diverged into three outputs so that the number and position of the interfaces in the main microchannel could be controlled. Each of the input and output channels were $50 \mu\text{m}$ wide, merging into and diverging from, respectively, a $150 \mu\text{m}$ wide, 20 mm long channel.

The main channel design involved a tradeoff between interfacial surface area and stability. The larger surface area of a longer channel promotes cell separation because there is more room and time for cells to sample each phase. However, the interfaces drift over the course of a long channel and become unstable near the exit of the channel. Drift was a problem because it redirected the interface to different outlet channels, which reduced separation efficiency. Tall channels introduced instability into the interface as well. Because the phases have different densities, the initially vertical interface became nonlinear near the exit of the main channel. After testing several device dimensions, we found that the instabilities limited the length of the main channel to 20 mm and the height to $100 \mu\text{m}$. The width of the main channel was not considered because the thickness of the center stream was controlled by the relative flow rates between the channels.

4 Methods

The two-phase system was prepared by combining PEG (Poly-Ethylene Glycol 8000, Fisher Scientific), Dex (Dextran MW 500,000, Fisher Scientific), $10\times$ PBS ($10\times$ Phosphate Buffered Saline, Sigma) and deionized water in a conical tube to create a 50 ml solution of $1\times$ PBS, 4% w/w PEG and 5% w/w Dex. The PBS was added to ensure the phases would remain physiologically osmotic, providing a final solution with 10 mM phosphate, 154 mM sodium chloride and a pH of 7.4. This concentration of polymers and salts was used for all experiments. The electrostatic potential generated by these salt concentrations was approximately $40 \mu\text{V}$ (Walter et al. 1976) which is far too small to induce movement in the blood cells during separation. The tube was shaken vigorously to break up large clumps of Dex and stored at room temperature overnight to ensure that the polymers were fully dissolved. PEG and Dex aliquots were extracted $500 \mu\text{l}$ at a time using 1 ml syringes. Dex was extracted from the bottom of the tube and PEG was extracted from the top of the tube.

The effect of interfacial area between phases on leukocyte concentration was investigated by characterizing stream configurations with zero, one, and two interfaces (Fig. 2). Zero interface conditions were achieved via two approaches. In the first configuration, all three streams contained PBS. The second configuration had a PEG-PBS-Dex stream pattern. PBS in the center channel diluted the polymers to approximately 67% of their original concentrations which caused the interface between the two-phases to disappear about 4 mm downstream of the input channels. In both experiments, whole blood was flowed in through the middle input channel and cells were collected at the middle output channel.

The one interface configuration was created with a PEG-PEG-Dex stream pattern. In this approach a 10 μl sample of whole blood was diluted into 500 μl of PEG solution and flowed through the middle input microchannel between streams of PEG and Dex. Input flow rates were adjusted via the syringe pumps so that the PEG-Dex interface exited into the center output channel, while minimizing the amount of Dex that was collected. The stream containing the blood sample had a flowrate of 1 $\mu\text{l}/\text{min}$ and the outer streams were adjusted as needed to position the interface, typically 0.7–2 $\mu\text{l}/\text{min}$.

The two interface configuration was created using a Dex-PEG-Dex stream pattern. Here, two outer streams of Dex were used to double the interfacial surface area that the blood cells suspended in the middle PEG stream were exposed to. Maintaining stable interfaces with two streams of high viscosity Dex required extra pumps at each of the Dex exit channels. The flow rates were adjusted so that the PEG stream, with interfaces, was collected in the middle output channel.

Blood samples were prepared similarly in all of the experiments. One hundred microliters of whole blood were taken from a finger prick of the same volunteer and placed in an EDTA coated tube (K2-EDTA Vacutainer, Becton-Dickinson, Franklin Lakes, NJ). The ratio of erythrocytes to leukocytes was counted each time with a hemacytometer. The variation of the initial erythrocyte to leukocyte ratio among samples for all experiments was less than 7%. Ten microliters of blood were then taken from the EDTA tube and added to the appropriate syringe and mixed. Cells were always introduced into the device via the middle input. During each experiment approximately 50 μl of effluent was collected from the devices by directing the middle outlet, via tubing, into 100 μl wells of a multiwell plate.

The erythrocyte and leukocyte populations were then counted on a hemacytometer. The hemacytome-

Fig. 2 (A) Whole blood is exposed to one interface, represented by the *dashed line*, in the PEG-PEG-Dex configuration. The leukocytes (WBC) prefer the interface while erythrocytes (RBC) migrate to the Dex. (B) The two stream interface increases the surface area that blood is exposed to, resulting in more effective leukocyte concentration

Table 1 Comparison of the separation efficiencies of zero, one, and two interface microscale and macroscale ATPS

	Control	Macro-scale	PBS only	No intf.	One intf.	Two intf.
RBC/WBC ratio	949 ± 64.9	707 ± 570	528 ± 247	594 ± 144	188 ± 107	104 ± 12.8
WBC enrichment	1.0 ± 0.07	1.34 ± 1.1	1.80 ± 0.84	1.60 ± 0.39	5.05 ± 2.87	9.13 ± 1.12
SAV (cm ⁻¹)	NA	1.27	0	0	100	400

A correlation exists between surface area-to-volume (SAV) ratio and the degree of leukocyte concentration. The fold-increase was calculated with respect to the unconcentrated control blood sample. All measured values are sample means plus or minus one standard deviation with $n = 3$.

ter and coverslip were cleaned with ethanol and Kim-wipes. After both pieces had dried, the coverslip was placed on hemacytometer and 10 μ l of cell solution was aliquoted into the hemacytometer. The cells were counted and recorded as erythrocytes. Lysing solution (Zap-O Globin, Beckman Coulter, France) was added to the remaining sample and counting was repeated and the cell count was recorded as leukocytes.

For the macroscale ATPS, 10 μ l of blood was placed in a 1.5 ml centrifuge tube containing 500 μ l of each phase, mixed well, and then allowed to sit for 30 min so the phases could separate. All experiments were performed three times ($n = 3$).

5 Results and discussion

Five different experimental setups were tested for their ability to separate leukocytes from whole blood (Table 1). For the control, cell counts were taken directly from whole blood in EDTA-coated tubes and had an erythrocyte to leukocyte ratio of 949 ± 64.9 which was within the normal range for a healthy adult human male.

The macroscale experiment in a static ATPS resulted in both the erythrocytes and leukocytes migrating to the lower bulk Dex phase. Cells were carefully collected from the Dex side of the interface. The average ratio of erythrocytes to leukocytes was lower compared to the control (707 ± 570), suggesting that the leukocytes preferred being near the interface, most likely due to their larger surface area. These results also support the findings discussed below in which higher leukocyte concentrations were found in samples of the PEG-Dex interface from the microfluidic devices.

To create a one-interface microfluidic setup a PEG-PEG-Dex stream pattern was used. Blood was mixed with PEG and flowed in through the middle stream. The blood cells were pinched against the Dex stream, greatly reducing the time required for them to sample both phases compared to the macroscale ATPS (Fig. 3(a) and (b)). Samples collected from this setup had an average erythrocyte to leukocyte ratio of 188 ± 107 , or a 5.05-fold increase in leukocyte concentration over the control.

A two interface system was created with a Dex-PEG-Dex stream pattern and resulted in collected samples with an average erythrocyte to leukocyte ratio of

Fig. 3 The one interface setup consisted of a PEG-PEG-Dex pattern. (A) Blood was introduced in the middle stream and (B) erythrocytes migrated to the Dex by the end of the channel. (C) Blood was introduced into the two interface device (Dex-PEG-Dex) which provided twice the surface area for erythrocyte migration, as shown in (D)

104 ± 12.8 , or a 9.13-fold increase over the control. The two interface system had an interfacial surface area double the one interface system and thus the interfacial surface area-to-volume ratio was four times greater than in the one interface system. This experimental setup created samples with slightly higher erythrocyte and leukocyte counts because the addition of a second interface increased the likelihood that blood cells in the middle stream would sample both phases (Figs. 3(c) and (d)). Microfluidic methods which can further increase the surface area-to-volume ratios between the two phases should result in even higher concentrations of leukocytes.

Zero-interface experiments were conducted in the microfluidic devices to determine the effects of hydrodynamic (i.e., non-interfacial) forces on leukocyte separation efficiency. In the first experimental configuration PBS was used in all three microchannels to quantify how flow alone affected erythrocyte and leukocyte concentrations in the middle collection channel. Compared to the control sample and macroscale ATPS, a lower erythrocyte to leukocyte ratio of 528 ± 247 was observed. The reduced ratio of erythrocytes to leukocytes suggests that the physical properties of blood cells and their behavior in laminar flow can be used to concentrate leukocytes since the erythrocytes spread out from the center stream faster than the leukocytes. The second zero-interface microfluidic experiment used a PEG-PBS-Dex stream pattern to test the separation efficacy of PEG and Dex without an interface. This setup produced two distinct interfaces at the beginning of the main microchannel which gradually disappeared about 4 mm downstream as the PBS diffused into the PEG and Dex streams. The PEG-PBS-Dex stream pattern resulted in a collected sample with an erythrocyte to leukocyte ratio of 594 ± 144 , or a 1.6-fold increase over the control. The separation in this arrangement is slightly less effective than with the zero-interface all PBS pattern, which proves that the polymers themselves do not cause separation and that a distinct interface between phases is required to significantly concentrate leukocyte populations within whole blood samples.

6 Conclusion

Microfluidic ATPS allowed us to create a precise, consistent fluidic environment with a stable interface in which we could carefully control the location of the cells and the position of the interface. More importantly, microfluidics allowed us to collect cells trapped at the interface, which is not practical with current

macroscale ATPS methods. The ATPS surface area-to-volume ratio was large enough to quickly expose cells to the interface and increasing the ratio in the device boosted separation efficiency. However, maintaining a stable interface required a separate pump at each input. The interfaces within microfluidic ATPS were sensitive to slight pressure variations and could easily be disrupted if the flowrate was too large (greater than $10 \mu\text{l/min}$) which limited the throughput of separated cells.

The purity of separated cells could be improved by cascading devices in series and the throughput could be increased by adding devices in parallel. The successful combination of both parallel and serial expansion would yield a device that could continuously separate significant volumes of cells with high specificity. However, very stable flowrates would be required to maintain a stable interface. Also, the cell medium would need to be reconcentrated after each separation since it gets diluted after each exposure to the interface.

The current device is targeted at separating white blood cells from whole blood, but any heterogeneous cell solution with a sufficiently high partition ratio can be fractionalized and even sub-fractionalized. For example, the serial devices need not perform the same separation. In such a configuration, each cascade would have a different two phase system that could pull out a different cell type from solution. These devices have the potential to be used in any application in which sufficiently differing cells need to be separated (e.g., isolating circulating cancer cells or detecting bacteria).

Microfluidics is an attractive approach for ATPS which allows the traditional drawbacks such as slow separation times and low surface-to-volume ratios to be overcome. In this work we have shown that concentrating leukocytes from whole blood by means of an ATPS in a microfluidic device is feasible, resulting in up to a 9.13-fold increase in the concentration of leukocytes. Future developments which decrease the erythrocyte to leukocyte ratio would make ATPS in a microfluidic device an attractive method for blood cell separation due to its biocompatibility and relatively innocuous effects on cells.

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