

## Separation of lipids from blood utilizing ultrasonic standing waves in microfluidic channels

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A method to continuously separate different particle types in a suspension is reported. Acoustic forces in a standing wave field were utilized to discriminate lipid particles from erythrocytes in whole blood. The presented technology proposes a new method of cleaning, *i.e.* removing lipid emboli from, shed blood recovered during cardiac surgery. Blood contaminated with lipid particles enter a laminar flow micro channel. Erythrocytes and lipid particles suspended in blood plasma are exposed to a half wavelength standing wave field orthogonal to the direction of flow as they pass through the channel. Because of differences in compressibility and density the two particle types move in different directions, the erythrocytes towards the centre of the channel and the lipid particles towards the side walls. The end of the channel is split into three outlet channels conducting the erythrocytes to the centre outlet and the lipid particles to the side outlets due to the laminar flow profile. The separation channel was evaluated *in vitro* using polyamide spheres suspended in water, showing separation efficiencies approaching 100%. The system was also evaluated on whole blood using tritium labelled lipid particles added to bovine blood. More than 80% of the lipid particles could be removed while approximately 70% of the erythrocytes were collected in one third of the original fluid volume. The study showed that the further reduced micro channel dimensions provided improved performance with respect to; (i) separation efficiency, (ii) actuation voltage, and (iii) volumetric throughput as compared to earlier work.

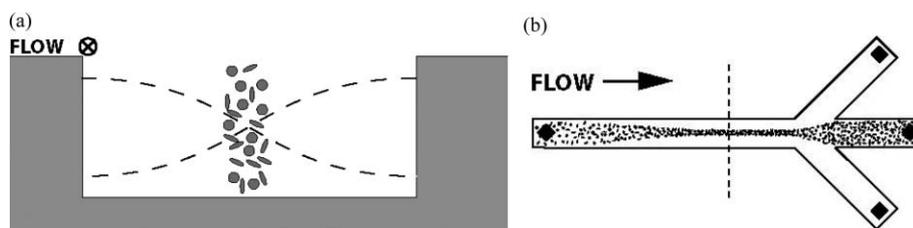
### 1 Introduction

Suspended particles in an acoustic standing wave field are affected by acoustic forces. The primary acoustic radiation force was thoroughly described for the first time by King in 1934.<sup>1</sup> King limited his work to incompressible spheres but his acoustic force theory was later extended by Yosioka and Kawasima<sup>2</sup> and by Gorkov<sup>3</sup> to include compressible spheres. Several groups have since used these theoretical results to increase the concentration of various particles in different media.<sup>4–10</sup> This has commonly been done by moving the particles into one fraction of the medium where after some of the particle free medium has been removed. However, there are two fundamental problems associated with the use of acoustic standing waves to separate particles. First of all the resonance cavity has to be very precisely fabricated to satisfy the resonance criterion. This leads to a difficult design and fabrication process. Secondly the cavity has to be coupled to an ultrasonic actuator in a way such that the power loss to the bulk material is minimised, which further increases the complexity of the separation device.

A separation cavity in silicon including improved means of coupling the acoustic actuator to the device has been proposed by Nilsson *et al.*<sup>4</sup> Briefly, micro channels were wet etched into a silicon wafer and sealed using anodic bonding. This resulted in very well defined structures and a fairly simple design and fabrication process. Silicon has also been used by Harris *et al.*<sup>9,10</sup> to fabricate separation cavities of a different design. Nilsson *et al.*<sup>4</sup> realized the ultrasonic actuation by applying a piezoceramic plate to the back-side of the silicon wafer using ultrasound gel. This actuation method resulted in good acoustic coupling of the ultrasound to the separation chip.

The width of the channel (750  $\mu\text{m}$ ) was chosen to correspond to one wavelength of the applied ultrasound. This gave rise to a standing wave, with two pressure nodes and three pressure anti-nodes. When 5  $\mu\text{m}$  polyamide spheres suspended in water entered the channel they moved into the pressure nodes and remained there throughout the length of the channel because of the laminar flow properties. The end of the channel was split into three outlets and the particles were collected *via* the side outlets while the main part of the particle free medium exited through the centre outlet. The results showed that 90% of the particles could be gathered in 2/3 of the original fluid volume.

This paper proposes the use of a smaller channel, 350  $\mu\text{m}$  wide, and a standing wave with only one pressure node. The purpose of using only one node is that particles can be gathered in the centre outlet while the main part of the medium exits through the side outlets. Also, the smaller channel width enables the use of the same high actuation frequency even though operating at a half wave length mode, which should provide a strong acoustic force on the particles. Another advantage is that two types of particles can be separated from each other if their physical characteristics are appropriate in relation to the carrier fluid. This effect can be used in a medical application to remove lipid particles from blood collected during open heart surgery. The lipid particles originate from adipose tissue that is a part of the dissection field. When shed blood is collected and returned to the patient lipid droplets formed by triglycerides originating from adipose cells are also introduced into the circulatory system. Since the lipid particles commonly are larger than the erythrocytes these are dispersed throughout the capillary network of the body causing massive embolisation of various organs, of which the brain is most sensitive. Lipid micro embolisation of the brain in conjunction



**Fig. 1** (a) Particles positioned, by the acoustic forces, in the pressure nodal plane of a standing wave. (Cross section of the channel in (b), dashed line.) (b) Top view of a continuous separation of particles, positioned in the pressure node, from a fraction of their medium.

to cardiac surgery have previously been reported<sup>11</sup> and linked to a temporary or permanent cognitive decay.<sup>12</sup>

## 2 Theory

### 2.1 Separation principle

Pressure fluctuations in a liquid medium result in acoustic radiation forces on suspended particles. As long as the diameter of the particles is much smaller than half the wavelength of the standing wave<sup>2</sup> these forces will act mainly in one direction and the particles will move towards either a pressure node, a pressure anti-node or not at all. The direction and size of the force can be estimated by the acoustic force theory<sup>13</sup> (eqn. (1)).

$$F_r = - \left( \frac{\pi p_0^2 V_c \beta_w}{2\lambda} \right) \phi(\beta, \rho) \sin(2kx) \quad (1)$$

$$\phi = \frac{5\rho_c - 2\rho_w}{2\rho_c + \rho_w} - \frac{\beta_c}{\beta_w} \quad (2)$$

The densities of the medium and particles are denoted  $\rho_w$  and  $\rho_c$  respectively and the corresponding compressibilities  $\beta_w$  and  $\beta_c$  respectively,  $p_0$  is the pressure amplitude,  $V_c$  is the volume of the particle,  $\lambda$  is the ultrasonic wavelength,  $\phi$  is a dimensionless constant defined by eqn. (2),  $k$  is defined by  $2\pi/\lambda$  and  $x$  is the distance from a pressure node. The direction of the force is determined by the sign of the  $\phi$ -factor, a positive  $\phi$ -factor results in a movement towards a pressure node and a negative towards a pressure anti-node correspondingly. For example, the theoretic value of the  $\phi$ -factor for polyamide particles in water is about 0.8 and hence they should move towards a pressure node.

The force described by eqn. (1) is referred to as the primary acoustic force. Understanding of the characteristics of the primary acoustic radiation force is sufficient to understand the acoustic particle manipulating concept of this paper but there is a group of other related acoustic forces that also influence the movement of the suspended particles. The secondary acoustic radiation forces and other relevant forces in this context have been thoroughly discussed by Ter Haar and Wyard<sup>14</sup> and Weiser *et al.*<sup>15</sup> The relative magnitude of these forces was also investigated in ref. 14.

If a micro channel is actuated at its fundamental resonance frequency, pressure anti-nodes will be present along the side walls of the channel and a pressure node along the centre. If the

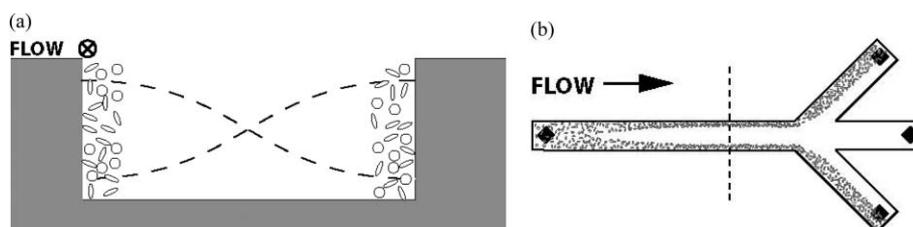
channel dimensions are small the flow will be laminar provided that the flow rate is reasonably low. When suspended particles enter the channel they will be affected by the acoustic forces and move towards either the pressure node (Fig. 1a) or the pressure anti-nodes (Fig. 2a) depending on the densities and compressibilities of the particles and the medium respectively. Since laminar flow conditions prevail, the particles will remain in their lateral positions even though being outside the acoustic field region until they reach the end of the channel, which is split into three different outlets. The particles can subsequently be collected in the centre outlet (Fig. 1b) or the two side outlets (Fig. 2b). By controlling the flow rate through the three outlets independently the fraction of the medium that exits together with the particles can be controlled.

### 2.2 Separation of particles with different physical properties

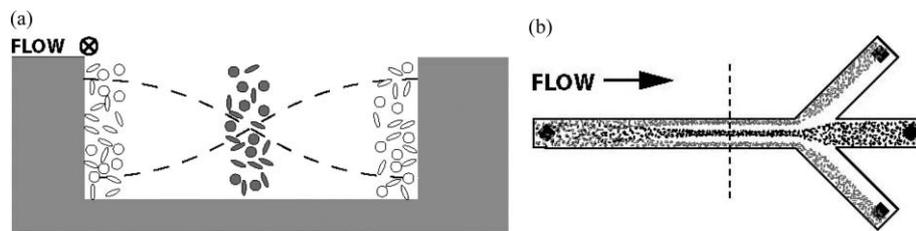
A consequence of eqns. (1) and (2) is that it in principle should be possible to separate two types of particles suspended in the same liquid medium from each other. If the  $\phi$ -factors (eqn. (2)) have opposite signs the two particle types will move in different directions in the acoustic force field. Eventually, one particle type will end up in the pressure node and the other type in the pressure anti-nodes (Fig. 3a). This effect can be used to separate different particle types in a continuous flow if the flow is laminar. If a half wavelength acoustic standing wave is used, one particle type will be located in the middle of the channel and the other along the side walls (Fig. 3b). When the channel is split into the three outlets one particle type will exit through the centre outlet and the other through the side outlets. For example, if the particles are erythrocytes and lipid particles (triglycerides) in blood plasma,  $\phi \approx 0.3$  and  $\phi \approx -0.3$  respectively, the erythrocytes should exit through the centre outlet while the lipid particles exit through the side outlets.

### 2.3 Downsizing

If two different particle types are to be separated from each other it is essential that the acoustic forces are sufficiently strong. Alternative ways to increase the acoustic force can be identified by considering eqn. (1), which tells us that one possibility is to increase the applied pressure amplitude. However, this can only be done to a certain extent since high pressure amplitudes also result in increased power dissipation and thus heating of the surrounding material and ultimately, cavitation and formation of gas bubbles in the system. Another



**Fig. 2** (a) Particles positioned, by the acoustic forces, in the pressure anti-nodal plane of a standing wave. (Cross section of the channel in (b), dashed line.) (b) Top view of a continuous separation of particles, positioned in the pressure anti-nodes, from a fraction of their medium.



**Fig. 3** (a) Two particle types positioned, by the acoustic forces, in the pressure nodal and anti-nodal planes of a standing wave. (Cross section of the channel in (b), dashed line.) (b) Top view of a continuous separation of two particle types from each other and/or a fraction of their medium.

alternative is to change the properties of the medium. This is more difficult and in some applications even impossible. The best way to increase the acoustic force is to decrease the ultrasound wavelength and the width of the separation channel accordingly.

### 3 Method and materials

#### 3.1 Micro machining

The separation chips were manufactured in silicon since the material displays good acoustic properties and the high precision fabrication process is well known. By using photolithography and anisotropic wet etching techniques a separation channel with perfectly vertical walls was obtained according to the method used in ref. 4. A structure with a 350  $\mu\text{m}$  wide and 125  $\mu\text{m}$  deep separation channel and a trifurcation outlet was designed (Fig. 4) and fabricated. A boron-silicate glass lid was attached to the silicon chip by anodic bonding to provide closed flow channels.

#### 3.2 Experimental arrangement

The piezoceramic element (PZ26, Ferroperm Piezoceramics AS, Kvistgard, Denmark) was powered by an in-house built sinusoidal signal power amplifier. The transducer was acoustically coupled to the rear side of the separation chip using ultrasound gel (Aquasonic Clear, Parker Laboratories Inc., Fairfield, NJ, USA). Silicone rubber tubings were glued to the inlets and outlets on the back side of the separation chip, acting as docking ports to the syringe pump (WPI SP260P, World Precision Instruments Inc., Sarasota, FL, USA) via standard 1/16" od teflon tubings. The flow rates through all three outlets were identical. The 5  $\mu\text{m}$  polyamide spheres used were suspended in a doppler blood phantom (Dansk Fantom Service AS, Jyllinge, Denmark) containing 20% particles. The bovine blood used was mixed with saline solution (9  $\text{mg ml}^{-1}$ , Fresenius Kabi Norge AS, Halden, Norway) in order to achieve different concentrations of erythrocytes. The lipid particles used were derived from a phospholipid-stabilized emulsion of triolein, which was prepared as described in detail

in ref. 16 with some modifications. Briefly, a total amount of 320 mg triolein, tritium labelled and unlabelled, and 3.2 mg of phospholipids were sonicated in 7.2 ml of PBS. Following sonication, 0.8 ml of 20% BSA in PBS was added. The lipid emulsions were always used within 5 h from the time of preparation. The degree of hemolysis was measured using a HemoCue Plasma/Low Hb meter (HemoCue AB, Ångelholm, Sweden).

To measure the fraction of particles (polyamide spheres or erythrocytes) recovered, *i.e.* the separation efficiency, samples from the centre and side outlets were collected in capillaries (Bloodcaps 50  $\mu\text{l}$ , VWR International AB, Stockholm, Sweden). Each sample was centrifuged (Haematokrit 2010, Hettich Zentrifugen, Tuttlingen, Germany) for 2 min at 13000 rpm after which the height of the particle pillars in the capillaries were measured, *A* (fluid collected from centre channel) and *B* (fluid collected from side channel). The separation efficiency was defined as  $A/(A + 2B)$ .

The lipid content was measured by a scintillation counter (Wallac Guardian 1414 Liquid Scintillation Counter, Perkin-Elmer Life and Analytical Sciences Inc., Boston, MA, USA), according to standard scintillation counting protocol,<sup>17</sup> with the modification of using Ultima Gold (Packard Biosciences, Boston, MA, USA) as scintillation liquid. The lipid separation efficiency was calculated in line with that of the other particle types.

## 4 Results and discussion

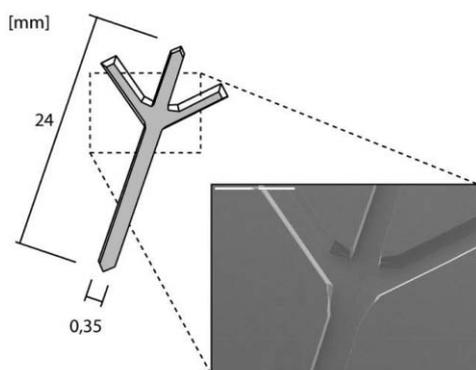
#### 4.1 Separator design

The earlier reported separation channel (750  $\mu\text{m}$  wide and 250  $\mu\text{m}$  deep) was operated in a single wavelength standing wave mode.<sup>4</sup> The new design (350  $\mu\text{m}$  wide and 125  $\mu\text{m}$  deep), allowed the system to use a half wavelength standing wave in the 2 MHz range for acoustic separation. The use of a single pressure node made it possible to collect the particles in 1/3 of the total fluid volume. Another improvement was the use of ultrasound gel as an interface between the piezo ceramic element and the silicon, instead of epoxy. The advantage of not gluing the transducer to the chip was the possibility to reuse the same transducer several times. The Reynolds number for the 350  $\mu\text{m}$  channel was calculated to 40, as compared to 20 for the earlier reported larger separation channel,<sup>4</sup> which still guarantees a stable laminar flow

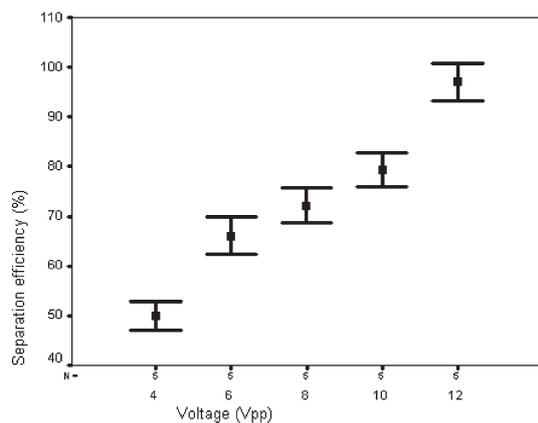
#### 4.2 Separator performance

As seen in Fig. 5, the particle separation efficiency was found to be very close to 100% at 12  $V_{pp}$  (voltage peak-to-peak). It can be noted that voltage needed was lower as compared to the earlier design yet obtaining a high separation efficiency. The fast decrease in separation efficiency as the voltage was decreased can be explained by the fact that the force is proportional to the square of the voltage, *i.e.* applied pressure.

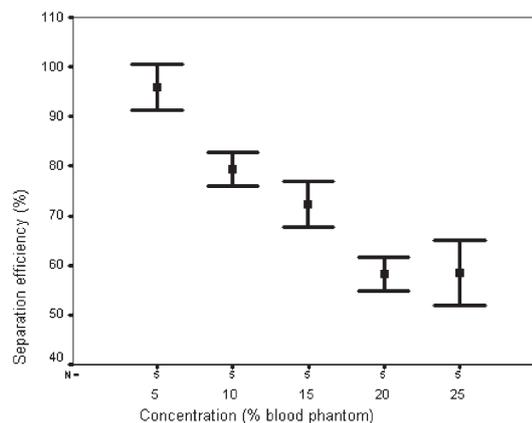
The flow rate tests (Fig. 6) showed that low flow rates resulted in high separation efficiencies. The reason for this was that the suspended particles were exposed to the ultrasound



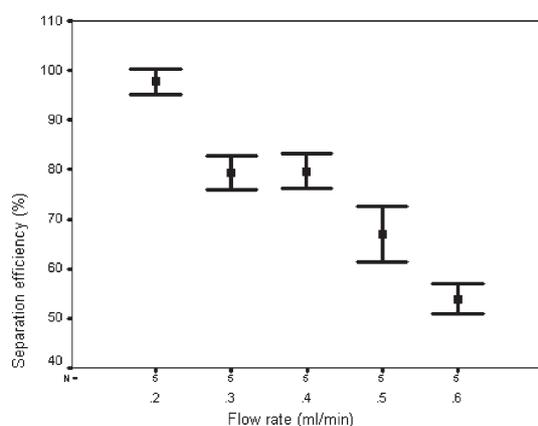
**Fig. 4** Schematic drawing and scanning electron microscopy image of the trifurcation region of the 350  $\mu\text{m}$  wide separation chip.



**Fig. 5** Separation efficiency of the 350  $\mu\text{m}$  structure versus voltage applied to the piezo ceramic element. The total flow rate was  $0.3 \text{ ml min}^{-1}$  and the blood phantom concentration was 10%.



**Fig. 7** Separation efficiency of the 350  $\mu\text{m}$  structure versus blood phantom concentration. The total flow rate was  $0.3 \text{ ml min}^{-1}$  and a voltage of  $10 \text{ V}_{\text{pp}}$  was applied to the piezo ceramic element.



**Fig. 6** Separation efficiency of the 350  $\mu\text{m}$  structure versus total flow rate. A voltage of  $10 \text{ V}_{\text{pp}}$  was applied to the piezo ceramic element and a blood phantom concentration of 10% was used.

field for a longer time period while travelling through the separation channel. The separation efficiency of the 350  $\mu\text{m}$  design was better than the 750  $\mu\text{m}$  design at the peak efficiency of the latter (between 95–100% compared to around 90%), even though the voltage was considerably lower ( $10 \text{ V}_{\text{pp}}$  compared to  $15 \text{ V}_{\text{pp}}$ ) and the flow rate was higher ( $0.2 \text{ ml min}^{-1}$  compared to  $0.1 \text{ ml min}^{-1}$ ).

At low particle concentrations very high separation efficiency was achieved (Fig. 7). The efficiency dropped as higher particle concentrations were investigated, mainly because a large number of particles demanded stronger acoustic forces to gather in well defined bands. Also, the centre outlet was overloaded by particles trying to exit the system that some particles were forced into the side outlets. It should also be commented that at higher particle concentrations secondary forces will eventually put a limit to the density of particles that can be accomplished in the centre of the channel as these will eventually counteract the primary force.<sup>15</sup>

#### 4.3 Separating lipids from blood

The average adult has four to six litres of blood in the circulatory system. Particles (erythrocytes, leukocytes and thrombocytes) compose about 45% of this volume and the rest is blood plasma. The plasma is composed of 90% water and 10% solved substances. The number of erythrocytes (red blood cells) is three orders of magnitude greater than the number of leukocytes (white blood cells) and more than ten times greater than the number of thrombocytes (platelets).

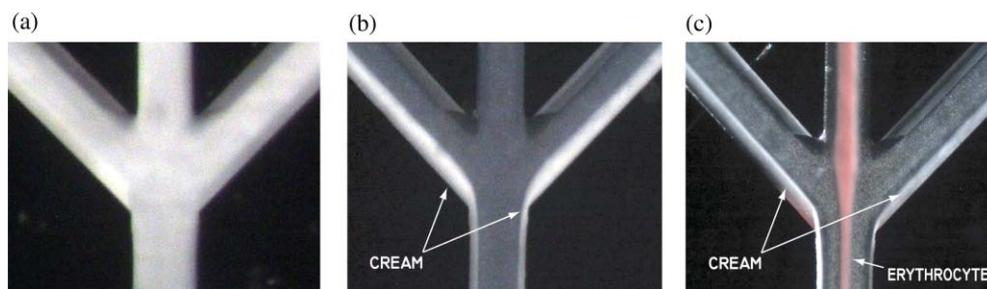
A major health care problem is the lack of allogeneic blood (donor blood). To reduce this demand shed blood can be collected and returned to the patient during or after surgery. There are several advantages associated with this method, called autologous blood recovery or blood wash. In addition to reducing the demand for allogeneic blood it reduces or eliminates transfusion transmitted disease and immunologic reactions to allogeneic blood. It also eliminates the risk of blood group incompatibility. Existing autologous blood recovery methods are based on centrifuges. A large volume of shed blood, typically 500 ml, is collected and centrifuged, the supernatant is removed and the collected erythrocytes are subsequently returned to the patient. However, this method suffers from three major shortcomings. First of all it can not remove lipids derived from surgery of adipose tissue efficiently and therefore offers no solution to the lipid emboli problem. Secondly, the erythrocytes experience high gravitational forces deforming them during the centrifugation process, and centrifugation is known to induce hemolysis.<sup>18</sup> Finally, the process is not continuous and demands a large volume of blood to initiate a cell wash cycle, which makes it inappropriate for many applications. The presented ultrasonic method is not burdened with these problems.

The different physical properties of erythrocytes and lipid particles yield a shift in sign of the  $\Phi$ -factor of eqn. (1), thus resulting in forces with opposite signs for the two particle types. This enables the gathering of lipid particles and erythrocytes at different locations in the acoustic standing wave field. Milk was used in initial tests (Fig. 8a – ultrasound off and 8b – ultrasound on) to confirm that lipid particles gathered in the pressure anti-nodes of the standing wave, along the side walls. A mixture of milk and blood was also successfully processed. When the ultrasound was turned on, the lipid particles moved towards the walls of the separation channel and the erythrocytes moved towards the centre (Fig. 8b) as expected.

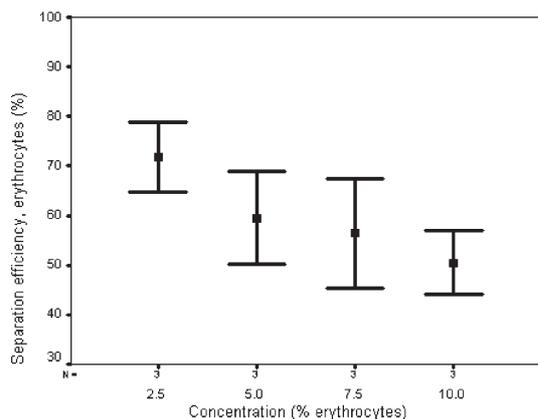
The separation efficiency of the chip was determined using tritium labelled triolein. The results showed that approximately 70% of the erythrocytes could be enriched in the centre outlet (Fig. 9) while more than 80% of the lipid particles were removed through the side outlets (Fig. 10). An interesting result was that the removed fraction of lipid particles was quite independent of the erythrocyte concentration.

The lipids occasionally formed clusters that tumbled along the channel walls, temporarily causing a decrease in erythrocyte separation efficiency since the laminar flow was disturbed and the straight flow lines of separated particles lost shape and destination.

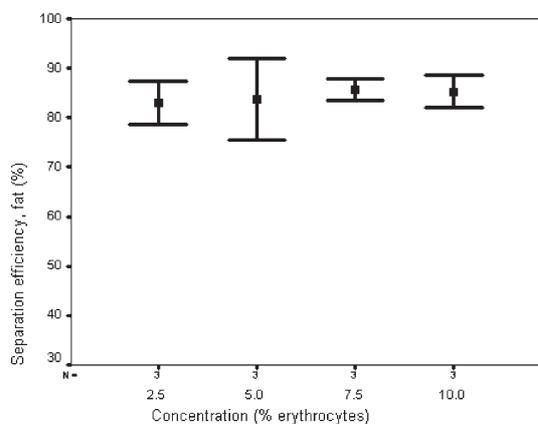
The tritium labelled triolein offered a possibility to evaluate



**Fig. 8** (a) Milk flowing through the 350  $\mu\text{m}$  separation chip with ultrasound turned off. (b) Milk flowing through the 350  $\mu\text{m}$  separation chip with ultrasound turned on. (c) A mixture of milk and blood flowing through the 350  $\mu\text{m}$  separation chip with ultrasound turned on.



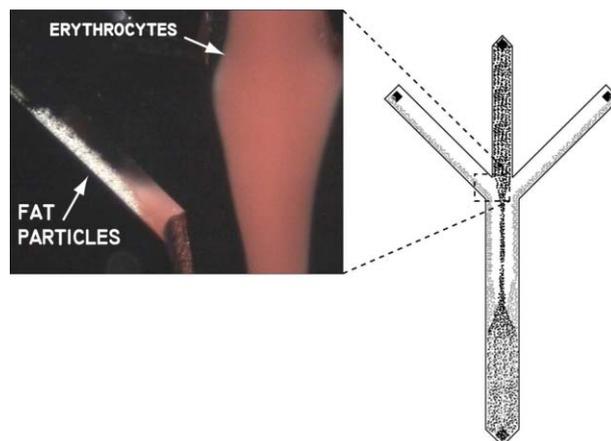
**Fig. 9** Separation efficiency *versus* erythrocyte concentration. The total flow rate was  $0.3 \text{ ml min}^{-1}$  and a voltage of  $10 V_{\text{pp}}$  was applied to the piezo ceramic element.



**Fig. 10** Separation efficiency of lipid particles *versus* erythrocyte concentration. The total flow rate was  $0.3 \text{ ml min}^{-1}$  and a voltage of  $10 V_{\text{pp}}$  was applied to the piezo ceramic element. All suspensions contained 1% lipids.

the lipid separation efficiency of the chip. Unfortunately, it was not possible to evaluate the chip's ability to remove lipids from human blood collected during cardiac surgery, because these lipids could obviously not be radioactively labeled. Neither could it be distinguished from the lipids contributed by lipoproteins upon measurement of total acylglycerides in plasma. However, a visual evaluation of the separation process indicated that it worked very well indeed (Fig. 11). The suspended lipid particles in blood collected during surgery are, in general, much larger than the lipid particles in the triolein emulsion used in this investigation and should be easier to remove since they are affected by stronger acoustic forces.

The degree of hemolysis was measured before and after separation and no increase was detected. This indicated that the separation process was gentle and harmless for the



**Fig. 11** Lipid particles separated from erythrocytes at the trifurcation of 350  $\mu\text{m}$  separation chip with ultrasound turned on.

erythrocytes. In this context it should also be mentioned that the ultrasound frequency used was within the frequency range used in diagnostic ultrasound and thus the risk of causing cell lysis by means of the applied ultrasound should not be a problem in the current setting.

## 5 Conclusions

It has been shown that the new design of the particle separator, 350  $\mu\text{m}$  wide, surpassed the earlier reported separator, 750  $\mu\text{m}$ , in terms of separation performance. A separation efficiency of almost 100% could be reached for polyamide spheres.

Most importantly, separation of two different particles types in a homogenous mixture was demonstrated. The fact that particles with different physical properties moved to different locations in the ultrasonic standing wave field, combined with the laminar flow micro channel with a trifurcation outlet, made this possible. Separation of erythrocytes from lipid particles was confirmed. In these initial experiments approximately 70% of the erythrocytes were recovered while more than 80% of the lipid particles were removed. These results in combination with the fact that no hemolysis was detected indicated that the method may be appropriate for the suggested intraoperative blood wash application.

Although the linear flow rate was increased in the new design, it was still insufficient for processing large volumes. A design with parallel separation channels for increased throughput is therefore desired.

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