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COMMUNICATION PAPER

Micromixing by pneumatic agitation on continually rotating centrifugal microfluidic platforms

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Abstract A highly effective pneumatic technique for mixing liquids on centrifugal microfluidic platforms is demonstrated and characterized. While a centrifugal platform is rotating, a stream of compressed gas is used to agitate liquids on the platform. This technique is implemented in a non-contact fashion and allows mixing without the need to alter the rotational frequency or direction of the centrifugal platform. Pneumatic agitation causes rapid mixing of the liquids and achieves homogeneity in 11.2 ± 1.2 s while rotating at 450 rpm (7.5 Hz), a 30-fold improvement compared to conventional mixing by interfacial diffusion. The mixing operation is shown to be equally effective when implemented over a range of rotational frequencies from 450 rpm (7.5 Hz) to 1,500 rpm (25 Hz).

Keywords Centrifugal · Microfluidic · Pneumatic · Mixing · Micromixing

1 Introduction

In recent years, interest in the field of microfluidics has grown exponentially, primarily due to the potential for performing rapid analyses and determinations for chemical and biological applications (Nolte 2009; Arora et al. 2010). Miniaturization of analytical systems in microfluidics allows analyses and diagnostics to be carried out with less labor and also decreases the amount of reagents or sample required. This is especially important in many biological applications where reagents or sample may be limited or expensive.

Centrifugal microfluidics, often known as "Lab on a CD", is a branch of microfluidics that primarily depends on the use of centrifugal force to pump fluids in a desired manner on the microfluidic platform (Madou et al. 2006). These microfluidic platforms are often shaped as disks and provide many advantages over conventional microfluidic platforms, especially in the development of micro-Total Analytical Systems (μTAS) (Gorkin et al. 2010). Using centrifugal force as the primary driving force means that these platforms do not need external pumps to make fluid flow, greatly reducing the number of physical connections that have to be made to the platform (Mark et al. 2010). These features make centrifugal systems convenient and simple to use for certain applications. Using centrifugal platforms, a large number of parallel analyses can be performed simultaneously as the centrifugal force acts uniformly across any given radius of the disk (Madou et al. 2001; Li et al. 2009).

In both conventional and centrifugal microfluidic systems, considerable effort has been dedicated to the development and enhancement of a wide range of unit operations such as liquid pumping, metering, and mixing (Kumar et al. 2011; Steigert et al. 2005). These operations form the basis for practical applications on these platforms. Of these unit operations, mixing remains one of the more challenging tasks. As mixing of multiple fluids in microfluidic structures is primarily diffusion limited, it can take a long time for homogeneity to be achieved, thereby degrading the efficiency of the entire microfluidic platform (Wang and Li 2007; Salmanzadeh et al. 2011).

A variety of ways has been reported to improve the efficiency of liquid mixing on centrifugal microfluidic

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platforms. Conventional diffusive mixing in chambers that are several millimeters wide typically takes place on a timescale of minutes (Steigert et al. 2005), while enhanced mixing methods usually reach homogeneity within several seconds. The most common of these techniques is known as "shake-mode mixing", whereby the direction of rotation of the centrifugal platform is repeatedly changed (between a clockwise rotation and a counter-clockwise rotation) (Grumann et al. 2005; Steigert et al. 2005). The splashing action of the liquid due to the frequent reversal of rotational direction causes mixing. However, a consequence of shake-mode mixing is that the disk is momentarily stationary when the rotational direction is altered, potentially allowing liquid to flow in undesirable ways (e.g., priming of siphons, backflow into capillary channels). Other mixing methods that have been described include mixing using Coriolis force (Haeberle et al. 2005) or by generating reciprocating flow between two chambers (Noroozi et al. 2009). However, these methods have certain constraints on the required rotational frequency or the design of the disk itself. A highly effective method of mixing involves the use of paramagnetic beads on the disk, which work with several stationary magnets to induce a stirring effect in the mixing chamber of the disk (Grumann et al. 2005; Ducrée et al. 2007). This method greatly decreases mixing time, but requires a more complex fabrication processes for both the disks and the spin platform.

In previous reports, we have demonstrated that an externally applied pneumatic gas flow can enable some important unit operations on centrifugal platforms including flow switching (Kong and Salin 2011a) and quantitative transfer as well as adding new capabilities such as pumping against centrifugal force (Kong and Salin 2010). This pneumatic technique relies on a regulated stream of compressed gas positioned directly above the vent holes of microfluidic chambers. Vent holes are necessary features in most centrifugal microfluidic structures to allow for liquid flow. As the disk rotates, air pressure is exerted on liquid in these chambers via the vent holes at a frequency based on the rotational frequency of the disk. Adapting that concept, we report here a non-contact mixing method that uses pneumatic pressure to cause agitation of a body of fluid, resulting in rapid mixing. This provides an additional capability for pneumatically supplemented centrifugal microfluidic platforms, expanding the toolbox of techniques available to researchers in this field. This pneumatic mixing technique does not require that the disk be stopped and is very tolerant with respect to the rotational frequency used. The added design features are trivial in both complexity and space required. The mixing efficiency of this technique is quantitatively verified and compared with conventional diffusive mixing. The efficiency of shake-mode mixing using our platform design is also verified.

2 Experimental

2.1 Platform fabrication

A polycarbonate centrifugal microfluidic platform was designed using 3-D computer-aided design (CAD) software Solidworks 2005 (SolidWorks Corp., MA, USA) and fabricated using methods described in detail by LaCroix-Fralish et al. (2009). A schematic of the disk design with dimensions is shown in Fig. 1. Both chambers were 1.4 mm deep, and the channels for vents were 0.7 mm deep. A 0.1 mm deep burst valve was cut into the adhesive layer between the top and bottom chambers to separate the liquids at the beginning of the experiment. Commercially available red and blue food coloring dyes were used for evaluating the mixing efficiency of this technique.

2.2 Image acquisition

The experiment was carried out using a pneumatic instrument configuration previously described (Kong and Salin 2011b). A schematic depicting this instrument configuration is shown in Fig. 2. To evaluate the mixing performance, high-speed digital images were obtained using the enhanced motor and strobe system previously described (Duford et al. 2009; Kong and Salin 2011a). The motor, strobe, camera and solenoid valve were controlled using a custom

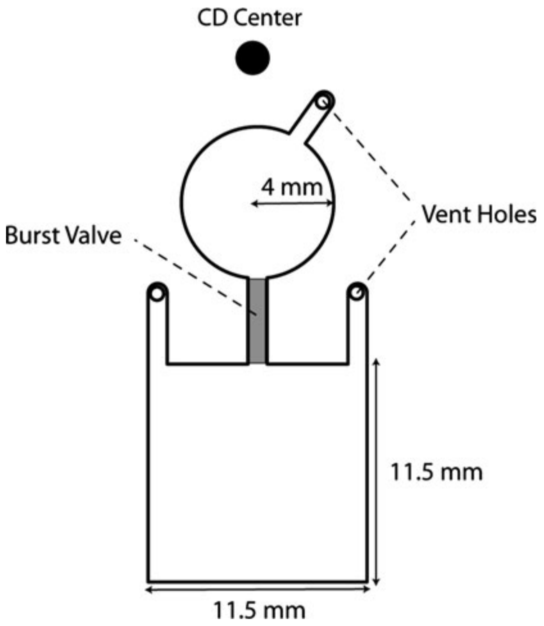

Fig. 1 Schematic of the disk design used to conduct the mixing experiment. The burst valve was experimentally evaluated to burst at ~400 rpm. The two air vent holes at the *top* of the mixing chamber were designed to be at the same radial distance from the *center* of the disk

LabVIEW program (LabVIEW 8.6, Developer Version, National Instruments, QC, Canada). The disk was arbitrarily spun in a clockwise direction for all experiments.

2.3 Procedure: diffusive and pneumatic mixing

To evaluate *diffusive mixing*, the disk was preloaded with 60 µL of red dye solution in the mixing chamber and 60 µL of blue dye solution in the top chamber and a spin cycle was initiated at 300 rpm for 10 s, followed by 450 rpm for 360 s. The blue dye solution took about 7 s (from $t = 11$ to 18 s) to empty into the mixing chamber at the bottom as the burst valve only allowed fluid flow at rotational frequencies above 400 rpm.

To evaluate *pneumatic mixing* the same type of disk was loaded with the same reagents and run as described above. The airflow (for agitation) was initiated 18 s after the start of the spin cycle to allow all the blue dye solution to empty into the mixing chamber. The airflow rate used was 1.0 SCFM (standard cubic feet per minute) ($\sim$28 L min^{-1}), a flow rate determined experimentally to be suitable for this configuration. This flow rate was experimentally determined to enable efficient mixing while not causing liquid splashing or destabilization of the rotating disk.

As the disk rotates, the two air vents connected to the mixing chamber are sequentially exposed to the stream of compressed air, as shown in Fig. 3. Pulses of pressurized air cause the dye mixture to be agitated resulting in rapid mixing.

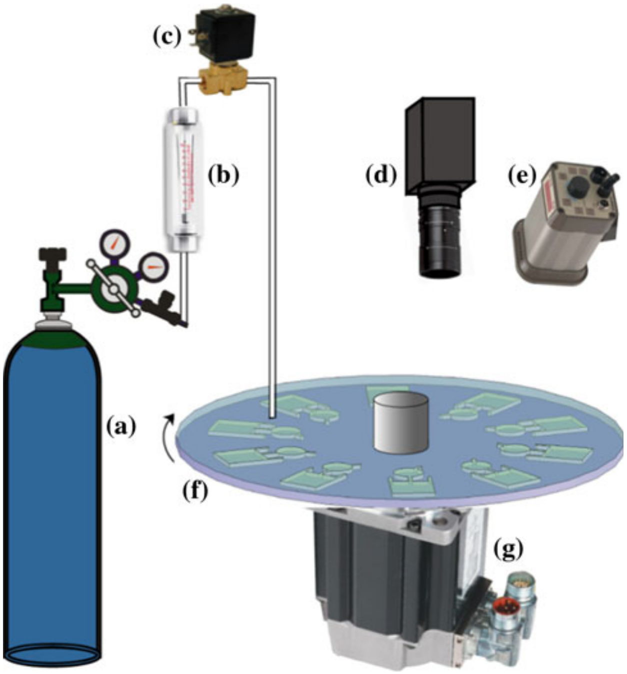

Fig. 2 Schematic of the experimental instrument configuration. **a** Cylinder of compressed air. **b** Rotameter to measure the flow rate. **c** On/off solenoid valve to control the air supply. **d** Camera and lens. **e** Strobe light **f** Centrifugal platform used for evaluating the mixing performance. **g** Servo motor

2.4 Procedure: shake-mode mixing

To verify the mixing efficiency of shake-mode mixing, the same type of disk was preloaded with the same reagents as above. A spin cycle was initiated at 450 rpm for 8 s, followed by the shake-mode time period, followed by another 10 s at 450 rpm. Several different durations of 14, 8, 5, and 3 s were used for the shake-mode time period, at a rotation-reversal frequency of 150 rpm (2.5 Hz).

2.5 Procedure: effect of rotational frequency on pneumatic mixing

The functionality of pneumatic mixing was then investigated and compared at additional rotational frequencies including 450, 600, 750, 900, 1,050, 1,200, 1,350, and 1,500 rpm. These spin cycles were conducted for 3 min at each rotational frequency, with an airflow at 1.0 SCFM configured to begin at 8 s into the spin cycle.

2.6 Image analysis

High-speed digital strobed images were obtained using a color CCD camera (GRAS-14S5C-C, Point Gray, BC, Canada), and analyzed using a custom MATLAB program (MATLAB R2011b, MathWorks Inc., MA, USA). Visually, the effectiveness of pneumatic mixing can be monitored based on whether distinct layers of red and blue dye are present, or if a homogeneous purple mixture is observed. However, the extent of mixing was rigorously quantified by defining a digital (pixel) region of interest (ROI) in the mixing chamber. Then, a "mixing index" was computed based on the gray intensities of each pixel's red, green and blue colors. These gray intensities are commonly known as pixel RGB values. Mixing indices are very commonly used in the literature to quantify the extent of mixing, and several variations of them can be found (Cha et al. 2006; Wang and Li 2007; Salmanzadeh et al. 2011).

For our quantitation, we adopted the mixing index defined by Wang (Wang and Li 2007), calculated as

$$\varepsilon = 1 - \sqrt{\frac{(R_{\mathrm{obj}} - R_{\mathrm{ref}})^2 + (G_{\mathrm{obj}} - G_{\mathrm{ref}})^2 + (B_{\mathrm{obj}} - B_{\mathrm{ref}})^2}{(R_{\mathrm{pur}} - R_{\mathrm{ref}})^2 + (G_{\mathrm{pur}} - G_{\mathrm{ref}})^2 + (B_{\mathrm{pur}} - B_{\mathrm{ref}})^2}} \qquad (1)$$

where R_{obj}, G_{obj}, and B_{obj} are the gray intensity values of the red, green and blue colors, of an object pixel from an experimental ROI. R_{pur}, G_{pur}, and B_{pur} are the corresponding gray intensity values for pixels of a purely

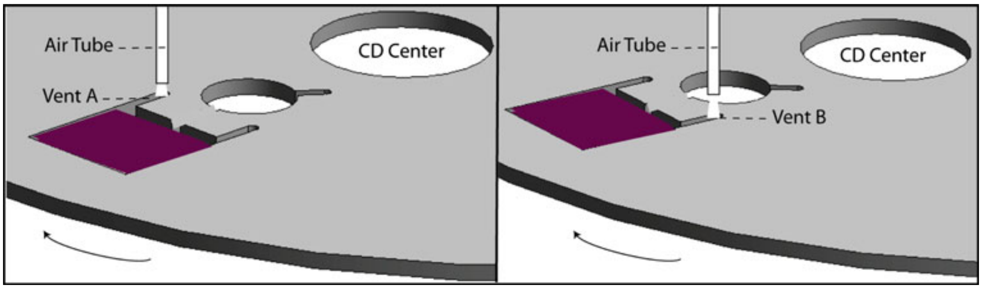

Fig. 3 Cut-away schematic depicting how pneumatic mixing is achieved. (*Left*) As the centrifugal platform rotates in a clockwise direction, *vent A* is first exposed to the air pressure. (*Right*) Disk rotation causes *vent B* to be exposed to the air pressure. The sequential pulses of air agitate the liquid causing a swirling action and results in rapid mixing

unmixed dye (either pure red or pure blue). R_{ref}, G_{ref}, and B_{ref} are the corresponding gray intensity values for pixels of a thoroughly mixed solution of red and blue dye. Ideally, a pixel from an image of unmixed dye will yield a mixing index of 0, while a pixel from an image of well-mixed dyes will yield a mixing index of 1.

For each experimental image, we defined a rectangular ROI of 8,875 pixels and calculated a root-mean-squared (RMS) mixing index, defined by

$$\varepsilon_{\text{RMS}} = \sqrt{\frac{\sum_{i=1}^{n} \varepsilon_i^2}{n}} \tag{2}$$

where ε_i is the mixing index of the *i*th pixel, and *n* is the total number of pixels. In an ideal situation, the value of ε_{RMS} is expected to be close to 0 if liquid in the ROI is unmixed, and close to 1 if liquid in the ROI is well mixed.

3 Results and discussion

3.1 Diffusive and pneumatic mixing performance

The performance of the pneumatic mixing technique is based on the sequential pulses of air causing agitation of the liquid body, resulting in a swirling wave-like movement of the liquid. Visually, as seen in Fig. 4, it is clear that pneumatic mixing significantly decreases the time it takes for the mixture to reach a homogeneous state as compared to static diffusive mixing. It takes approximately 12 s of exposure to pneumatic flow for the red and blue dye solutions to be mixed thoroughly, while diffusive mixing still results in obvious layers of red and blue dye solutions even after 172 s. A video depicting the effectiveness of pneumatic mixing can also be found in Online Resource 1.

A detailed quantitative analysis further showed that it takes about 6 min (360 s) for the dyes mixed by diffusion to reach the same "extent of mixing" achieved in 11.2 ± 1.2 s ($n = 3$) of exposure to the pneumatic flow. This is shown in Fig. 5. This indicates a 30-fold improvement in the mixing performance, demonstrating the potential for decreasing analysis times and greatly improving the mixing capabilities of liquids on centrifugal microfluidic platforms.

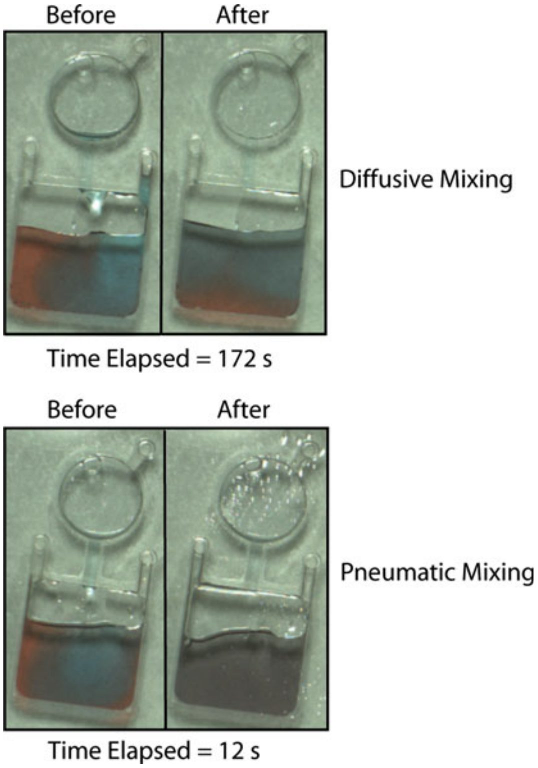

Fig. 4 Experimental strobed images demonstrating the effectiveness of pneumatic mixing. (*Top*) Distinct layers of *blue* and *red* dye can still be seen clearly even after 172 s of diffusive mixing. (*Bottom*) The *blue and red* dyes are thoroughly mixed after 12 s of exposure to pneumatic agitation, resulting in a *purplish* mixture (color figure online)

As seen in Fig. 5, thorough mixing appears to be achieved when the calculated mixing index approaches 0.92, instead of the expected 1. By using a series of reference (thoroughly mixed) images as experimental images, further calculations verified that the true experimental upper limit of the mixing index is 0.92. This is due to the noise associated with the pixel RGB values used to

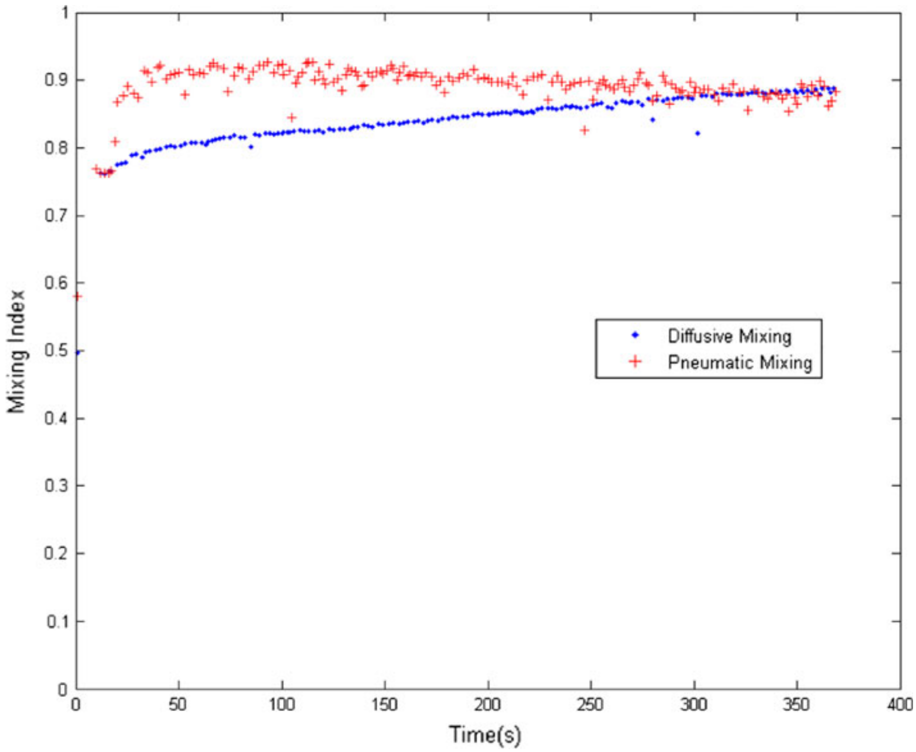

Fig. 5 Comparing the calculated mixing indices from experimental strobed images during diffusive mixing and pneumatic mixing over a 370 s spin cycle. Addition of the *blue* dye solution to the *red* dye solution was initiated 10 s into the spin cycle by increasing the rotational frequency to 450 rpm. Pneumatic flow was initiated 18 s into the spin cycle. It takes about 6 min of diffusive mixing to achieve the same extent of mixing as 12 s of pneumatic agitation. This indicates a 30-fold improvement in mixing efficiency (color figure online)

calculate the mixing index (Eq. 1). Even slight deviations between the experimental and reference pixel RGB values will result in ε_{RMS} having a value <1. To unambiguously differentiate between "mixed" and "unmixed" states, the means and standard deviations of the mixing indices for each state were calculated. After pneumatic mixing, the mixing index was 0.897 ± 0.017, whereas before pneumatic mixing, the mixing index was 0.764 ± 0.003. As the values of the mixing indices are very different, it was possible to properly quantify the mixing performance of the pneumatic method.

A major advantage of this pneumatic mixing technique is that it does not require major structural design features on the disks or the integration of foreign entities such as magnets or beads, as it only requires air vent holes that are often a necessity on centrifugal microfluidic platforms. This greatly simplifies disk designs and offers flexible experimental conditions—an important aspect if many sequential, complex steps are to be integrated onto a single platform. Another advantage to mixing by pneumatic agitation is its applicability to centrifugal platforms rotating at low rotational frequencies. Often, it is desirable for unit operations such as mixing to occur at low rotational frequencies, because fluid movement to the next step of the analysis may be designed to occur at higher rotational frequencies (e.g. when using capillary burst valves). Since capillary burst valve frequencies are typically above 500 rpm (Cho et al. 2007), mixing at a rotational frequency lower than that is unlikely to trigger unwanted fluid flow. If initial unit operations must take place at very high rotational frequencies, controlling fluid flow during a later phase of the experiment becomes more difficult. A potential concern regarding the use of an external pneumatic system is contamination or evaporation of samples. However, in our experiments, there are no observable evaporation effects, which is probably due to the small area that

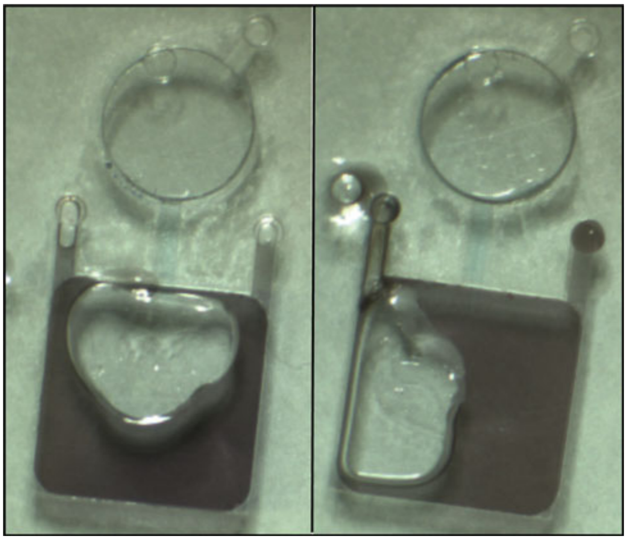

Fig. 6 Strobed images of shake-mode mixing illustrating the potential for back flow through channels and out of vent holes. (*Left*) Backflow of liquid through channels is possible due to the frequent reversal of rotational direction and momentary loss of centrifugal force. (*Right*) During shake-mode mixing, liquid could splash out of vent holes, potentially leading to sample loss

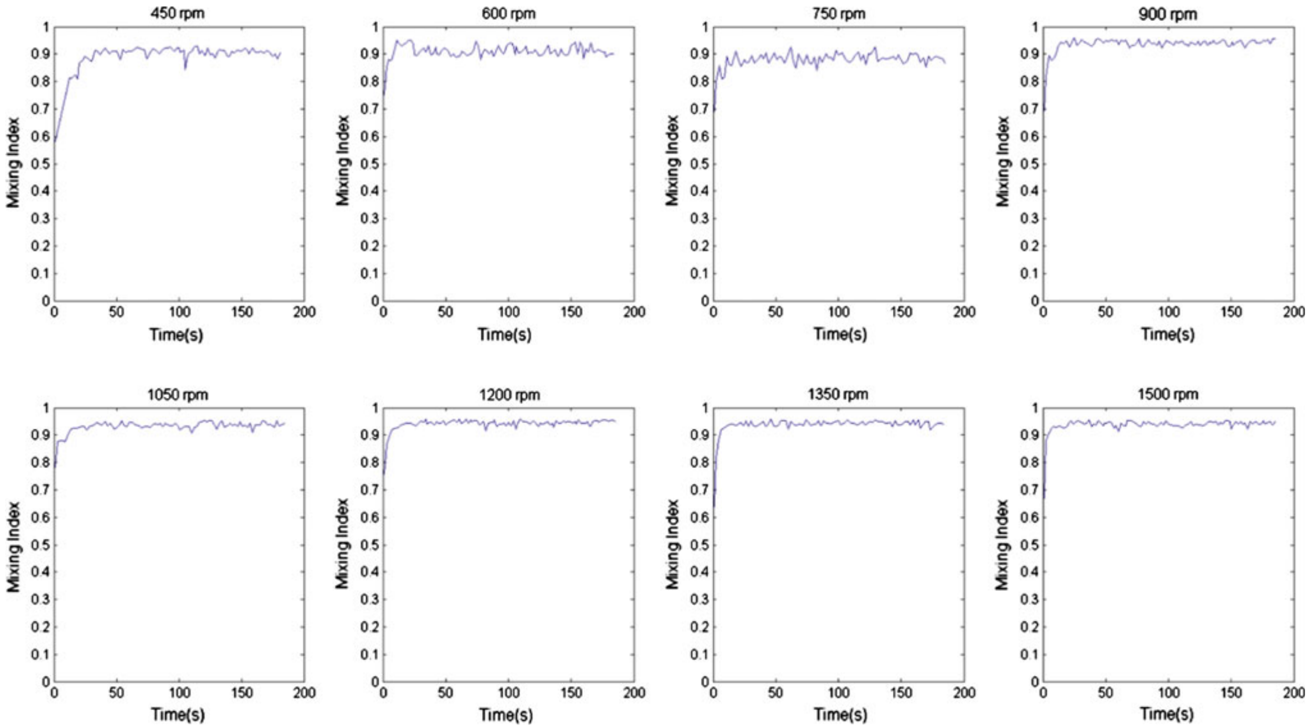

Fig. 7 Mixing indices for pneumatic mixing conducted over a range of rotational frequencies of the centrifugal platform over 3 min spin cycles. Pneumatic mixing performance is maintained throughout the investigated range of rotational frequencies

the liquids are exposed to. The problem of contamination can also easily be eliminated by using high purity, filtered air.

If higher mixing rates were necessary, specially designed mixing chambers that increase turbulence and promote mixing might also be integrated onto the platform, thereby increasing the mixing efficiency and decreasing the amount of time required to reach completion.

3.2 Shake-mode mixing

Shake-mode mixing has been established as an effective and widely used technique in the field of centrifugal microfluidics, previously reported to achieve thorough mixing in about 3 s (Steigert et al. 2005). Our experiments verified that thorough mixing was achieved when our disk was put through 3 s of shake-mode mixing. However, a significant disadvantage to shake-mode mixing is the requirement that the disk be momentarily stopped every time the direction of disk rotation is reversed. This might be undesirable if there are microfluidic features such as siphons, a useful structure, in the disk. Siphons may be primed when the disk is stopped and reversed during shake-mode as the momentary loss of centrifugal force may cause liquid to flow backwards through channels. Abrupt changes in rotational frequency may also cause liquid to splash out of air vent holes. An example of this can be seen in Fig. 6. Pneumatic mixing can be applied while the centrifugal platform is continuously rotating in a single direction, ensuring that a constant centrifugal force is applied on the liquid body, consequently preventing unwanted backflow of liquid into channels or siphons. In addition to the presence of a constant centrifugal force, it is observed that pneumatic agitation is much gentler than shake-mode mixing, as illustrated by the swirling action of the liquid. This minimizes the chance for liquids to flow backwards or prime siphons during pneumatic mixing. Although pneumatic mixing is not as fast as shake-mode mixing, it reaches completion within a reasonable timescale (12 s) and should not significantly degrade the throughput of centrifugal microfluidic platforms.

Comparing pneumatic and shake-mode mixing, pneumatic mixing provides an alternative mixing technique with its own set of advantages and disadvantages. Although it requires an external pneumatic system and is slightly less efficient than shake-mode mixing, it allows for milder agitation with greater control over the liquid body as centrifugal force is never lost. Eliminating the possibility of liquid backflow and splashing allows for greater experimental flexibility and simpler platform designs.

3.3 Effect of rotational frequency

As the rotational frequency of the centrifugal platform increases, the amount of time that the liquid is exposed to air pressure per revolution decreases. However, the number of pulses of air that the liquid is exposed to during the spin cycle increases. The performance of pneumatic mixing was assessed at rotational frequencies between 450 and 1,500 rpm, at 150 rpm intervals. As seen in Fig. 7, increasing the rotational frequency does not appear to diminish the effectiveness of this pneumatic mixing technique. At every rotational frequency, thorough mixing is achieved after approximately 12 to 14 s of exposure to air.

4 Conclusions

The pneumatically driven technique presented here provides an efficient, non-contact way of mixing liquids on centrifugal microfluidic platforms, adding another important capability to the repertoire of previously demonstrated pneumatic operations. Compared to static diffusive mixing, pneumatic agitation was capable of achieving thorough mixing of liquids on a timescale short enough to be useful in fast microfluidic multi-operation sequences. Pneumatic mixing provides several advantages over established mixing techniques that are commonly applied to centrifugal systems. This pneumatic mixing system can potentially be integrated with other centrifugal microfluidic operations very easily as it is almost independent of disk designs and rotational frequencies.

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