AGAROSE DROPLET MICROFLUIDICS FOR HIGHLY PARALLEL SINGLE MOLECULE EMULSION RT-PCR

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ABSTRACT

A microfluidic device for generating uniform agarose droplets for highly parallel single molecule emulsion reverse transcription PCR (eRT-PCR) is presented. The device mixed RNA template and RT-PCR reagents, in agarose solution, immediately prior to mono-disperse droplet generation to ensure uniform concentration and distribution of components. After eRT-PCR, the agarose droplets were solidified by cooling to form stable beads enabling downstream processing whilst maintaining monoclonality. Stochastic data were then obtained using fluorescence flow cytometry to demonstrate detection of single copy number RNA within agarose droplets. This method allows highly efficient, high throughput, eRT-PCR suitable for gene expression studies, and other applications.

KEYWORDS: Agarose Droplet Microfluidics, Reverse Transcription Polymerase Chain Reaction, Flow Cytometry, RNA

INTRODUCTION

RT-PCR is a commonly applied amplification technique well suited to the study and detection of low copy number RNA. It can be particularly useful for single cell gene expression studies, viral load studies and viral diagnostics and also for RNA sequencing applications. When used in conjunction with quantitative, "real-time" PCR (qPCR) it can be especially powerful for quantitative and highly sensitive detection of low abundance RNA [1-4]. Further combining the technique with the highly mono-disperse droplet emulsion techniques made possible by microfluidic devices, it can provide powerful systems for rapid and high-throughput analysis and can allow efficient RNA sequencing [2, 5].

In previous work we have developed the use of microfluidic droplet generators to produce highly mono-disperse agarose droplets for emulsion PCR (ePCR) [6]. The distinctive sol-gel properties of agarose allow for highly efficient ePCR without the need for microbeads since, after thermally induced switching to the gel phase, the droplets form stable agarose microbeads which are able to maintain the monoclonality of each droplet. Such beads are also sufficiently mechanically robust to allow centrifugation and strong agitation without disrupting their integrity. Additionally, since there is no need to compartmentalize a single microbead in each reaction droplet, there is a much greater efficiency of generating positive droplets.

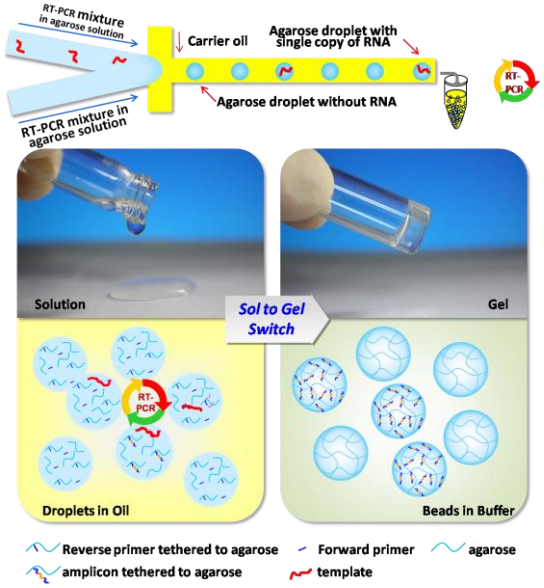

Figure 1: Schematics of the agarose emulsion droplet microfluidic method for single copy RT-PCR. Statistically diluted templates are encapsulated into uniform nanoliter agarose-in-oil droplets, which are then thermally cycled for RT-PCR amplification. Each agarose droplet contains free forward primer, while reverse primer is attached to the agarose. Droplets with RNA template will produce amplicons physically trapped within the agarose after eRT-PCR. Following eRT-PCR, the droplets are cooled to gelate to agarose beads for downstream genetic analysis

Using microfabricated droplet generators to produce agarose droplets also allows high throughputs with more than 500 droplets per second possible from a single generator. The homogeneity of the agarose PCR solution provides no negative effects such as steric hindrances and charge repulsion associated with solid support microbeads. This allows highly efficient amplification particularly for long amplicons and is thus very beneficial for DNA/RNA sequencing. More importantly, the high monodispersity and ability to tune the size of droplets allows effective control over the reaction volume and the amount of dNTPs which is vital for effective, quantitative applications including single cell RT-PCR. Furthermore, after solidification of the agarose droplets, further processing such as FACS analysis and sequencing becomes much easier.

In this work we demonstrate for the first time, the use of agarose droplets for performing single molecule eRT-PCR and apply flow cytometry to demonstrate single copy RNA amplification. A simple microfluidic device was designed and fabricated to allow even distribution of RT-PCR reaction components within highly monodisperse and size tunable droplets. Statistical dilutions could easily be performed to demonstrate low copy number RT-PCR. After performing RT-PCR, droplets were cooled to form stable beads and stochastic analysis performed using fluorescence flow cytometry.

EXPERIMENTAL

Figure 1 shows an overview of the microfluidic agarose droplet RT-PCR scheme used. A two-inlet glass microfluidic chip was designed for agarose droplet generation. An agarose solution containing RNA sample template is pumped from one inlet while the enzymes and other components required for the RT-PCR reaction are injected from the other. The reverse primer had previously been tethered to the agarose matrix by first synthesizing 5'-acrydite primer and then grafting to linear polyacrylamide (LPA). This allowed the primer to become entrapped within the agarose polymer matrix and effectively prevent diffusion of RT-PCR reaction products. The forward primer was free in the agarose solution.

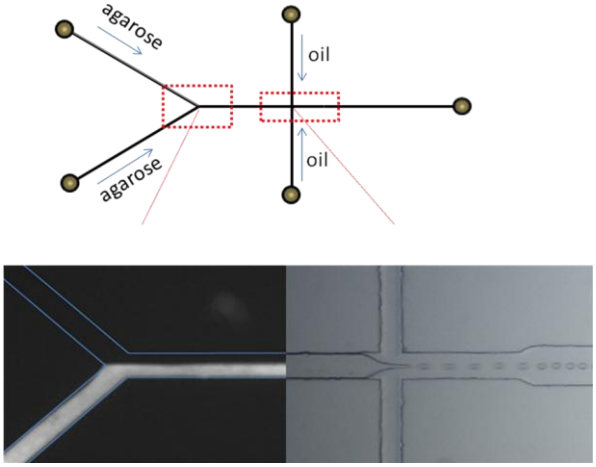

Figure 2: Top: Chip design showing two inlets, one for agarose solution plus RNA template, the other for agarose solution plus RT-PCR reagents. Bottom left: Laminar flow streams merge together to evenly mix agarose solutions containing template and PCR reagents (fluorescence used to enhance visualization). Bottom right: Agarose droplet generation using two oil inlets. The frequency of droplet generation is 300 Hz.

Figure 2 shows the microfluidic chip design. The two agarose solutions are brought together in a laminar flow stream and allowed to mix evenly by diffusion. A fluorescence microscope image visualizing the laminar flow is shown in the bottom left of figure 2. The flow then reaches a cross junction which injects a focusing flow of the oil phase, at which point monodisperse agarose droplets are generated (Figure 2, bottom right). This method ensures even mixing and uniform concentrations of all reagents within each droplet. After droplet generation, reverse transcription reaction and PCR are performed within the agarose droplets. After amplification, the droplets are cooled to 4 ℃ in order to gelate the droplets into stable agarose beads. Subsequently the agarose beads are analyzed using a flow cytometer for a range of different copy numbers per droplet.

RESULTS AND DISCUSSION

Figure 3 (left) shows flow cytometry results for a range of copy numbers per bead. The control (0 copies per bead) shows a single population of low fluorescence beads indicating no amplification. As the copy number is increased, so does the population of fluorescent beads indicating amplification. Figure 3 (right) shows a histogram comparing the measured and theoretical probabilities (calculated according to a Poisson distribution) for different copy numbers. The results indicate that high-throughput, high efficiency RT-PCR could be performed in agarose droplets with low abundance (single molecule) RNA.

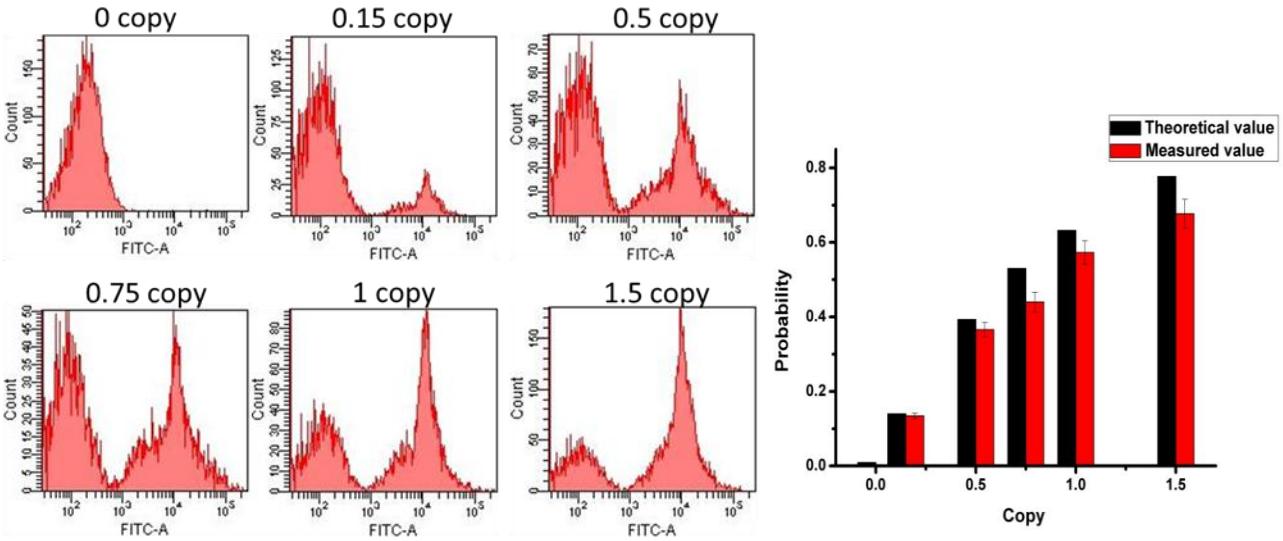

Figure 3: Left: Fluorescence flow cytometry results of single copy agarose droplet RT-PCR. From top left to bottom right: Control (0 copies), 0.15, 0.5, 0.75, 1.0, 1.5 copies per droplet. Right: The theoretical value (black) was calculated according to Poisson distribution and the observed value (red) was the statistic result according to the experimental data.

CONCLUSION

In this work we have clearly demonstrated single molecule, high-throughput, eRT-PCR using agarose microfluidic droplets. The use of a simple microfluidic device to evenly mix RT-PCR reagents into mono-disperse, size controllable agarose droplets, allows highly efficient amplification. The ability to thermally switch the agarose to stable beads enables easy downstream processing whilst maintaining monoclonality. We believe this will open up new possibilities for applications including single cell gene expression studies, viral load analysis and RNA sequencing.

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