Agarose droplet microfluidics for highly parallel and efficient single molecule emulsion PCR†‡

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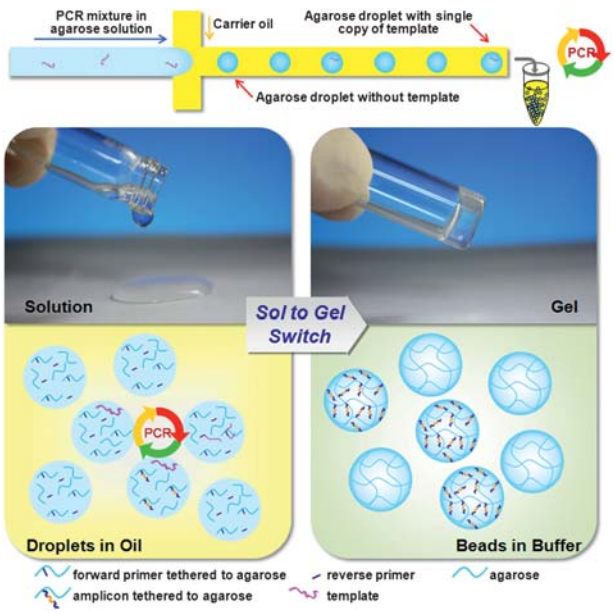
An agarose droplet method was developed for highly parallel and efficient single molecule emulsion PCR. The method capitalizes on the unique thermoresponsive sol–gel switching property of agarose for highly efficient DNA amplification and amplicon trapping. Uniform agarose solution droplets generated *via* a microfluidic chip serve as robust and inert nanolitre PCR reactors for single copy DNA molecule amplification. After PCR, agarose droplets are gelated to form agarose beads, trapping all amplicons in each reactor to maintain the monoclonality of each droplet. This method does not require cocapsulation of primer labeled microbeads, allows high throughput generation of uniform droplets and enables high PCR efficiency, making it a promising platform for many single copy genetic studies.

Compartmentalization of individual samples in aqueous droplets dispersed in an oil phase is becoming a powerful method for high throughput assays in chemistry and biology.[1–9] For example, by segregating single DNA molecules in individual water-in-oil emulsion droplets, emulsion PCR (ePCR) offers the advantage of massively parallel clonal amplification of DNA templates, which allows the identification and quantitation of rare mutant gene within a large population[1,10–12] and enables new generation of ultra-high throughput DNA sequencing technologies.[4,13,14] Conventional ePCR methods generate emulsion droplets with sizes ranging from femtolitre to picolitre by aggressively stirring[15,16] or agitating[1,4,12] the aqueous and oil phases together, where a single primer-immobilized microbead is encapsulated with a single DNA molecule in individual droplets. The DNA template is amplified on the microbead in the same droplet, which prevents the possibility of the mixing of DNA products from different droplets after lysis of emulsion droplets. The use of microbeads to maintain monoclonality is critical for downstream high-throughput manipulation and analysis. For example, genetic information of individual DNA templates encapsulated in droplets can be queried by analyzing their clones on each individual microbead by means of chemiluminescence or fluorescence. Although widely used, these methods are fundamentally limited because of several reasons. First, these approaches cannot efficiently and uniformly amplify long DNA amplicons needed for complex genome assemblies due to the extreme polydispersity of the droplets.[17] Second, quantitative single cell mRNA expression study cannot be performed in such polydispersed droplets given the sensitivity of RT-PCR to the amount of the reactants. Third, the use of microbeads leads to many problems including poor PCR efficiency and short product length because PCR on a solid surface suffers from problems like steric hindrance effect and charge repulsion.[18] Furthermore, by encapsulating diluted molecules and microbeads into the droplets at random, the resulting Poisson statistics leads to a large number of void droplets including empty droplets or droplets with only either molecule or microbead, which is wasteful, negating the speed and efficiency afforded by ePCR.[19] To address these challenging problems, herein we propose an agarose emulsion droplet microfluidic method for highly efficient and uniform ePCR.

Scheme 1 shows our agarose emulsion droplet microfluidic method. We use a microfabricated emulsion generator to produce highly uniform monodispersed nanolitre emulsion droplets of an agarose solution with PCR reagent in carrier oil. The droplet generator consists of a microfabricated nozzle[17] for controlled



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

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injection of a stream of agarose solution into a sheath oil flow. By adjusting the flow rate of aqueous and oil phase, precise control of the emulsion droplet size can be achieved. DNA template molecules are introduced into the PCR mix in a statistically diluted concentration so that on average there will be no more than one template in one droplet. The role of agarose in the aqueous phase is to serve as a capturing matrix to replace conventional primer functionalized microbeads to preserve the monoclonal nature of the DNA product in each droplet. We chose ultra-low gelling agarose, which has a melting point of about 56 °C and a gelling point around 16 °C. Once melted, this agarose will remain in the liquid phase until the temperature drops below 16 °C, which ensures easy generation of agarose droplet on chip under room temperature. In addition, at all PCR temperatures, agarose remains in liquid phase, where PCR can take place with high efficiency. On the other hand, after PCR amplification, the solution form of the agarose droplet can be switched to the solid gel phase by simply cooling the solution below gelling point. Once solidified, agarose beads will remain solid unless the temperature rises above 56 °C. As a result, DNA products amplified in the droplet will retain their monoclonality even after the oil phase is removed. The clonal beads can be used for downstream sequencing or genotyping applications.

We first checked the feasibility of generating agarose emulsion droplets on chip. Fig. 1A shows uniform agarose droplets can be consistently generated on chip at room temperature at a frequency higher than 500 Hz. As shown in Fig. 1B, droplets collected show a highly uniform diameter of 180 ± 7 μm that corresponds to a volume of 3.0 nL with a variance of only 0.3 nL. The size of droplet is tunable by controlling the microfluidic-chip channel dimension, flow rate of aqueous or oil phase. For example, using a chip with an oil channel of 360 μm and aqueous channel of 360 μm in width, uniform agarose droplets at the range of 110 to 200 μm can be easily obtained (Fig. 1E). Such a monodispersity is very important to ensure uniform PCR amplification for sequencing or other quantitative genetic analysis.

The agarose droplets generated are very thermostable. After being subjected to 30 rounds of rapid thermocycling, no change of size and monodispersity were observed. When the temperature dropped to 4 °C, agarose emulsion droplets solidified. Fig. 1C shows uniform agarose microbeads in oil at room temperature after cooling agarose emulsion droplets to 4 °C. The agarose beads were stable and remained in solid form at room temperature. Fig. 1D shows an array of agarose beads in water after the oil phase was removed. The mechanical strength of the agarose beads is strong enough to survive regular stirring and centrifugation during the washing process. Strong mechanical strength of microbeads affords flexible downstream processes and ensures monoclonality of each bead.

PCR efficiency in different concentrations of agarose solution was studied to further evaluate the feasibility of our agarose emulsion droplet PCR approach. Lambda DNA was used as the template for PCR (Reverse primer: CAA GCT TTG CCA CAC CACG GTA TT, Forward primer: TAA GCA CGA ACT CAG CCA GAA CGA). When agarose concentration was higher than 4%, PCR efficiency dropped below 80% (see ESI‡), which was likely due to the slow diffusion rate of reactants because of the high viscosity nature of the high agarose concentration. However, when agarose concentration was below 3%, no observable decrease in PCR efficiency was found, suggesting the feasibility of performing highly efficient PCR in low concentration agarose.

After establishing the feasibility of forming agarose emulsion droplet on chip and performing PCR in agarose, we performed PCR in individual agarose droplets. To prevent diffusion of PCR product from agarose beads, forward primer was conjugated to agarose matrix using a Schiff-base reaction as shown in Fig. 1F. 2% agarose droplets with 1.5 copies of DNA and PCR reagents were collected in a PCR tube. After 25 rounds of thermocycling, the droplets were cooled to 4 °C. The resulting agarose beads were collected by removing the oil phase *via* sequentially washing with acetone, isopropanol, and deionized water. After staining by SYBR Green, a fluorescence image of the agarose beads was taken. Individual microbeads were brightly fluorescent (Fig. 2A). In contrast, control beads with no DNA template had low fluorescence intensity (Fig. 2B). The experiment clearly demonstrated that DNA can be amplified in agarose droplets.

We continually diluted the DNA template concentration in the PCR mix to further prove the feasibility of the method for single template amplification. Under all stochastic conditions tested, for example, at the single molecule conditions of 1.5, 0.5, 0.15 copy/droplet, bright fluorescent beads were observed, clearly indicating the capability of the method for single copy genetic amplifications. More importantly, the frequencies of positive reactions over a range of

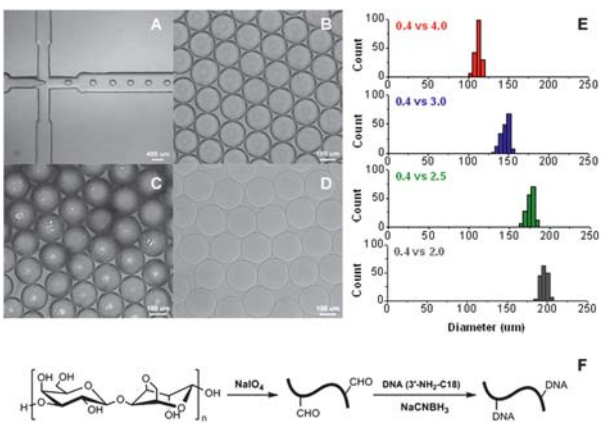

Fig. 1 (A) Generation of agarose droplets on chip. (B) Uniform droplets in oil before cooling. (C) Agarose droplets in oil solidified as beads after cooling. (D) Agarose beads in water. (E) Size distribution of agarose droplets with different oil flow rates (4.0 mL h^{-1}~2.0 mL h^{-1}) while keeping the flow rate of the aqueous phase at 0.4 mL h^{-1}. (F) Conjugation of forward primer to agarose matrix using Schiff-base reaction.

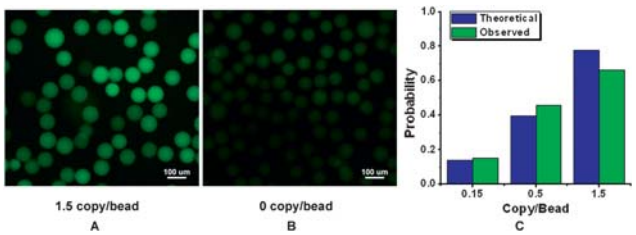

Fig. 2 Results of single copy agarose droplet PCR. Fluorescence microscope images of agarose beads after amplification from template concentrations of (A) 1.5 and (B) 0 copy/bead. (C) Percentage of microbeads carrying PCR product. The theoretical value (blue) was calculated according to Poisson distribution and the observed value (green) was the statistic result according to the experimental data.

template concentrations agreed closely with the frequencies predicted by Poisson statistics (Fig. 2C), demonstrating both the accuracy and sensitivity of this platform for limiting dilution and digital PCR applications.

Because polymerase chain reaction in agarose droplets is performed in the liquid phase, PCR efficiency is significantly higher than bead based ePCR where PCR efficiency suffers with its reaction carried out at the solid-liquid interface. To evaluate PCR efficiency in agarose droplets, the number of DNA generated in each individual agarose bead was quantified by Q-PCR. From a single template, an agarose droplet produced more than 10^6 copies of amplicon after 25 PCR cycles, suggesting that PCR efficiency as high as 95% can be obtained under agarose-in-oil droplet conditions. This number is remarkably higher than the PCR efficiency of about 40% we reported for microbead ePCR.[17]

In conclusion, we have developed an agarose droplet microfluidic approach for highly parallel and efficient single copy ePCR. Our method capitalizes on the unique thermoresponsive sol–gel switching property of agarose to avoid the use of microbeads in ePCR, which offers several advantages. First of all, droplets are monodispersed and size-tunable. This is very important for quantitative applications such as running reversed transcriptase polymerase chain reaction to compare the number of a particular mRNA among individual cells, where product is sensitive to the volume of the reactor and amount of dNTPs. Secondly, our agarose droplets significantly increase the efficiency of generating effective droplets because there is no need for the compartmentalization of limiting diluted microbeads. Thirdly, the throughput of the method is extremely high. A single generator can produce more than 500 droplets per second, making it a great platform for massive single copy genetic analysis. More importantly, by performing PCR in a homogeneous solution, the steric effect and charge repulsion in solid phase PCR are eliminated, leading to a high amplification efficiency, especially for those long amplicons that are of great significance to DNA sequencing application. Finally, agarose droplets, once solidified, change to agarose beads and can be easily processed and used for downstream applications such as sequencing, FACS analysis or long term storage *etc.* The agarose droplet method reported here allows uniform, massively parallel, highly efficient monoclonal amplification and holds great potential for a variety of applications such as single cell expression study, rare mutant detection, as well as next generation high throughput sequencing.

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