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Surfactant and oil formulations for monodisperse droplet emulsion PCR†

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Emulsion PCR has become a popular and widely applied method in biological research and clinical diagnostics to provide evenly amplified products and perform highly quantitative counting of target sequences. However, there is still a lack of information to support further development of appropriate water-in-oil emulsion formulations, which need to be both thermally and mechanically stable for digital amplification reactions. Here, we present a systematic survey of the oil and surfactant components of stable monodisperse w/o emulsions suitable for use with our previously developed micro-capillary array (MiCA)-based centrifugal emulsion generation method. Our findings show that a binary formula consisting of isopropyl palmitate and a silicone copolymer demonstrated the best performance, and provided a general guideline for the development of emulsion systems for digital PCR and emulsion amplification applications.

Introduction

DNA quantification and sequencing have revolutionized the way researchers study molecular biology and medicine and are now routine processes in many fields, particularly in fundamental genetics and genomics researches, clinical diagnostics, environmental and biological safety inspections, and various biotechnology industries.[1–3] DNA can be duplicated and then amplified faithfully *in vitro via* many polymerase-mediated reactions, such as thermal-cycling based polymerase chain reactions (PCRs),[4] and many isothermal amplification chemistries, such as multiple displacement amplification (MDA)[5] and loop-mediated isothermal amplification (LAMP).[6]

In addition to providing a large number of DNA products through massive duplication, amplification reactions also yield information that can be used to detect and quantify DNA molecules. Quantitative PCR has become a conventional assay for quantifying the copy number of target DNA molecules or fragments with specific sequences.[7,8] Recently, digital PCR (dPCR), which distributes a bulk PCR reaction into a large number of separate reactions and digitally counts the positive and/or negative results of each individual reaction compartment, has been proven to be a highly sensitive and accurate method for performing absolute counting of DNA copies.[9–14] In the next generation sequencing (NGS) applications, most input samples need to go through a DNA amplification-based process to construct a molecular library for subsequent sequencing. Similar to digital PCR, distributed amplification of DNA fragments, such as emulsion PCR (emPCR)[15] and emulsion whole genome amplification (eWGA)[16,17] approaches, have been developed to facilitate library construction in various NGS applications.

Emulsion amplification requires the distribution of DNA fragments within aqueous droplets in oil. Two major approaches, mechanical and microfluidic, have been widely applied in emulsification. It is challenging to achieve a highly uniform emulsion through mechanical vortexing,[15,18–20] and typical microfluidic emulsification may require specific instrumentation and unconventional operation protocols.[21–25] We previously reported a novel method for generating monodisperse water-in-oil (w/o) emulsion droplets using a laboratory centrifuge and micro-channel arrays (MiCA), and demonstrated emulsion dPCR and eWGA with greatly reduced engineering effort.[14,26] Through these studies, we have found that the formulation of oil and selection of surfactants that suit eWGA may not be appropriate for dPCR. Unlike eWGA,

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which is an isothermal process and generally requires mild reaction temperatures, dPCR requires the emulsion droplets to be stable throughout dozens of thermal cycling processes, repetitively reaching a high temperature of around 95 °C.

A universal formulation to suit all possible droplet generation methods is not yet available. In this work, we explore a wide range of surfactant and oil formulations, and test their performance by generating droplets using both microfluidic chips and MiCA, in an attempt to provide a general guideline along with some specific formulas for various droplet emulsion applications. In particular, we focus on finding practical oil–surfactant formulations for monodisperse emulsion PCR, specifically for digital PCR, which is probably the most valuable clinical application of such emulsions. Our evaluation is based on three major droplet characteristics: thermal stability, mechanical robustness, and monodispersity. The search of a specific oil–surfactant formulation for MiCA can inspire the design of other droplet-related techniques. Beyond just providing an oil–surfactant formulation for a stable emulsion that well serves our purpose, we also try to set a practical framework for the optimization of such formulation by providing an empirical theory and measuring standards.

Results and discussion

We primarily focused on relatively popular and prominent techniques for emulsion generation. The major parameters we explored were the intrinsic physical properties of the oil, particularly viscosity and density, as well as the availability of matched surfactants. In practice, although simple mechanical stirring, vortexing, or shaking can provide w/o emulsions for various biochemical reactions, poor monodispersity remains a significant problem that hinders their application in quantitative assays such as digital PCR (dPCR) or emulsion whole genome amplification (eWGA). Microfluidic approaches, which generally use chip-based devices using either T-junctions,[21,22] flow-focusing crosses,[24] or step emulsification,[27,28] have become the mainstream solutions for providing highly uniform w/o emulsion droplets with controlled size. One of the major advantages of microfluidic chip-based approaches is the wide choice of oils with different density and viscosity, as long as they are compatible with the chip materials. However, the operation of microfluidic chips can be a technically challenging task for many biological or clinical researchers and laboratories. MiCA has shown great potential to fill the gap between microfluidics and conventional lab operations[14] (Fig. 1a). However, the droplet formation schemes of MiCA and microfluidic chips are different and require particular consideration when choosing appropriate oil and surfactants.

The viscosity of the oil phase is critical to droplet generation. For microfluidic chips using a T-junction or flow focusing cross, the continuous aqueous phase is segmented into droplets through liquid interfacial shear force that periodically pinches the aqueous phase. Therefore, the size of the droplets is subject to the viscosity of the oil phase. The greater the viscosity of the oil, the greater the shear force at a constant flow rate, and thus the smaller the droplets (Fig. 1b). Whereas in step emulsification, the size of the droplets is thought to be primarily controlled by the wettability of the nozzle, which dictates the contact angle of the fluid, and viscosity is a negligible parameter.[28] However, viscosity plays a different role in the MiCA approach. As droplets are formed at the air–water interface rather than the water–oil interface, viscosity has little influence on the droplet size. However, when the droplets are ejected and move into the oil phase, the impact of aqueous droplets on the air–oil interface is much greater when the oil viscosity is high, which can cause droplets to shatter (Fig. 1b).

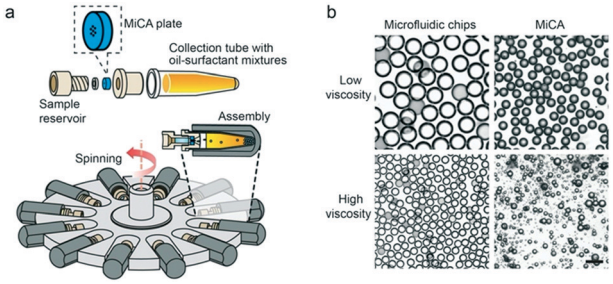

Fig. 1 MiCA-based technique for droplet generation. (a) An off-chip emulsion generation method that uses centrifugation with micro-capillary array (MiCA) devices. (b) The dispersity of the droplets depends on the viscosity of the emulsification oil. In conventional microfluidic-chip-based methods such as flow-focusing that rely on interfacial shear force, viscous oil results in smaller droplets; however, in the MiCA-based emulsion generation process, high viscosity can lead to droplet shattering. Low viscosity oil used for both microfluidic chips and MiCA: isopropyl palmitate supplemented with 7% Abil EM180. High viscosity oil used for microfluidic chips: 4.5% (v/v) Span 80, 0.4% (v/v) Tween 80, and 0.05% (v/v) Triton X-100 in mineral oil. High viscosity oil used for MiCA: 15% DEC, 15% mineral oil, and 70% silicone copolymer (SCP)-containing compound Abil WE09. Scale bar: 100 μm.

Our aim was to find a general guideline for choosing an appropriate formulation for PCR using the MiCA-based emulsion generation method. Although MiCA simplifies the hands-on operation in droplet generation, suitable oil–surfactant selection takes more consideration. Unlike chip-based microfluidic approaches in which oil density plays an insignificant role in droplet formation, in MiCA and many other non-chip methods, oil density is a major factor. For example, the widely used fluorinated oil cannot be used in MiCA as its density is greater than that of water so the droplets formed in air cannot penetrate the air–oil interface to form an emulsion during centrifugation. Similar considerations are also valid for spinning microcapillary droplet formation (SiMPLE)[29] and cross-interface emulsification[30] methods. For the MiCA approach, we found that oil with a density slightly lower than water is preferred. Under these conditions, the oil can offer just enough buoyancy to allow the droplets to sink to the bottom and not collide with droplets coming up, and to effectively buffer the sinking droplets from merging into a bulk phase.

Two major characteristics that we considered as indicators of the emulsion, which are highly associated with amplification performance, were the monodispersity and thermal stability of the droplets. High monodispersity typically yields much more uniform amplification efficiency between the compartments, leading to more even amplification across the whole genome in eWGA and higher precision of positive counting in dPCR.[14,26,31] High stability ensures that the droplets do not coalesce or rupture during the reaction, and hence it is a critical requirement for the accurate counting of digital PCR. Choosing surfactants that are compatible with the oil is key to these factors.[32] As a simplified model of amphiphilic surfactant molecules, the hydrophilic–lipophilic balance (HLB) value, which primarily relies on the molecular mass ratio of the hydrophilic groups of the surfactant, can be used to facilitate the understanding and selection of surfactants and their compatibility with the oil.

Three major categories of oil were studied: aliphatic oil, silicone oil, and fluorinated oil. We found that aliphatic oil has the greatest potential for MiCA droplet generation. Although fluorinated oil is typically chemically inert and highly compatible with biochemical reagents, making it a perfect fit for most reactions in aqueous droplets, the choice of suitable fluorinated surfactants is very limited.[33,34] Moreover, fluorinated oil is not suitable for centrifugal force driven droplet formation approaches due to its high density.[12,35,36] Most silicone oil has low density (*ca.* 0.9 g mL^{-1} at 25 °C), however its viscosity is too high for suitable droplet generation using MiCA. Some low viscosity silicone oils have been tested with various surfactants[37–39] and were found unable to form high monodispersity stable emulsions in a facile manner (ESI† Fig. S1).

We then focused on aliphatic oils, which have been coupled with non-ionic surfactants and used in various emulsion PCR applications (ESI† Table S1). Surfactants are key to stable emulsions as they adsorb onto the surface of droplets and lead to repulsive interactions that prevent coalescence when the droplets are in contact with each other. Many non-ionic surfactants are soluble in hydrocarbon oils, and are less sensitive to electrolytes, resulting in greater robustness in PCR reactions that commonly use buffers with high ionic strength. We tested five of the most popular formulations and found that for most experiments, the HLB values of the surfactants fall around 5, which agrees with the conventional understanding that oil-soluble surfactants with HLB values of around 4–6 will stabilize w/o emulsions.[40] We evaluated the emulsification performance using two approaches, a chip-based microfluidic generator and a MiCA generator, with a PCR buffer (20 mM Tris–HCl (pH 8.4), 50 mM KCl, and 5 mM $MgCl_2$) as the aqueous phase.

The thermal stability of the emulsion was investigated using a typical PCR process with 30 or 40 thermal cycles between 57 °C and 94 °C. Emulsions generated using oil mixtures supplemented with a silicone copolymer (SCP), such as cetyl PEG/PPG-10/1 dimethicone (Abil EM90) or an SCP-containing compound (Abil WE09, a mixture of cetyl PEG/PPG-10/1 dimethicone, polyglyceryl-4 isostearate and hexyl laurate), as the main active reagents show relatively better thermal stability (Fig. 2). These surfactants have been widely used in many reports.[18–20,41] One reason for this is that PEG/PPG and silicone-based surfactants tend to generate fewer bubbles. In addition, surfactants with silicone backbones as hydrophobic moieties typically further reduce the surface tension. Polymeric surfactants adsorb strongly at the water–oil interface and stabilize the emulsion droplets through steric effects. We found that oil mixtures containing bis(2-ethylhexyl) carbonate (or diethylhexyl carbonate (DEC), *e.g.* Tegosoft DEC) gave the best results (Fig. 2 and ESI† S2), suggesting that DEC was able to facilitate emulsion stability, similar to the previous report.[42]

We then investigated the multi-variable space by varying the compositions of mineral oil, SCP, and DEC for a suitable oil/surfactant formulation to form stable emulsions using MiCA. We specifically paid attention to the coalescence or splitting of the droplets during the experimental process, before and after PCR.

To achieve w/o emulsions, we focused on oil-soluble surfactants, particularly those that are preferentially soluble in the oil phase according to 'Bancroft's rule'.[43] Stable monodisperse emulsions could be obtained by adjusting the ratio of mineral oil, DEC, and SCP-containing compounds (*e.g.* Abil WE09). A broad range of each of these three components was initially tested (Fig. 3). Polymeric surfactants provide potent protection against the flocculation or coalescence of aqueous droplets, as well as Ostwald ripening, thus maintaining high monodispersity. Increases in temperature typically cause flocculation and coalescence due to the desorption of surfactants. Hence, the concentration of the surfactant cannot be too low when the emulsion is designed to be used at high temperature. However, if the proportion of the SCP-containing compound exceeds 30% and/or if the mineral oil exceeds 30% (Fig. 3a and b, conditions 1 and 2), many droplets with an exceptionally

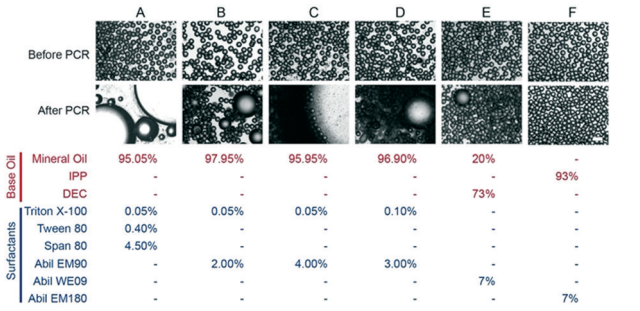

Fig. 2 Droplets generated with a PCR buffer (NEB) and reported aliphatic oil–surfactant mixtures using MiCA, observed before and after 40 PCR cycles. (A) 4.50% (v/v) Span 80, 0.40% (v/v) Tween 80, and 0.05% (v/v) Triton X-100 in mineral oil. (B) 2.00% (v/v) Abil EM 90 and 0.05% (v/v) Triton X-100 in mineral oil. (C) 4.00% (v/v) Abil EM 90 and 0.05% (v/v) Triton X-100 in mineral oil. (D) 3.00% (w/w) Abil EM 90 and 0.10% (w/w) Triton X-100 in mineral oil. (E) 7% (v/v) Abil WE 09, 20% (v/v) mineral oil and 73% (v/v) DEC. (F) 7% (v/v) Abil EM 180 in IPP. IPP: isopropyl palmitate; DEC: diethylhexyl carbonate; scale bar: 100 μm.

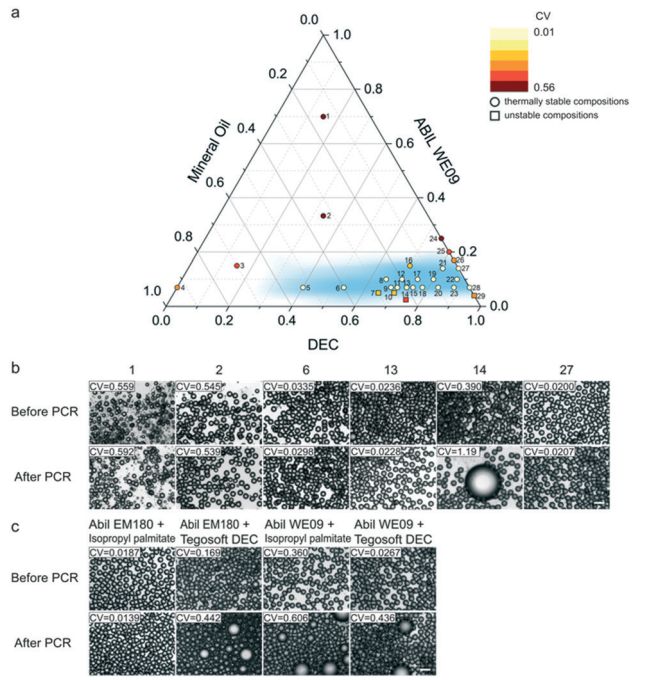

Fig. 3 The stability and monodispersity of w/o emulsion droplets generated by MiCA centrifugation. (a) The oil–surfactant composition survey for droplet generation. The aqueous phase was PCR buffer and the base oil was mineral oil and DEC, with an SCP-containing compound (Abil WE09) as the surfactant. In the mineral oil/SCP/DEC 3-component phase diagram, each dot represents an experimental composition that we investigated. The blue region indicates the formulas that can provide droplets with both high uniformity and high thermal stability. (b) Oil surfactant mixtures with different compositions indicated with numbers were tested with PCR buffer (NEB) through 30 thermo-cycles. Increasing the amount of SCP-containing compound or mineral oil resulted in numerous droplets with an exceptionally small size. The coalescence of droplets became severe if the SCP-containing compound was less than 5% (v/v). (c) Oil–surfactant mixtures of 7% (v/v) SCP-containing compound (Abil WE09) or SCP (Abil EM180) with DEC or isopropyl palmitate were tested with more PCR cycles (40 thermo-cycles). The results verified the high stability and good monodispersity of the emulsion generated using isopropyl palmitate supplemented with 7% (v/v) SCP (Abil EM180). Scale bar: 100 μm.

small size can be observed under a microscope. This is likely due to the high viscosities of the surfactant and mineral oil (viscosity of approximately ~50 cSt for mineral oils), which cause the aqueous droplets to break when they hit the air/oil interface. Such emulsions with poor size uniformity are not suitable for dPCR or eWGA. As anticipated, reducing the amount of the SCP-containing compound or mineral oil (Fig. 3a and b, conditions 6, 13, 14 and 27) effectively lowers the viscosity of the oil phase to around 10 cSt, and consequently helps to prevent droplets from splitting into smaller ones. As an extreme example, when we completely removed the mineral oil (Fig. 3a and b, condition 27), droplet splitting was also eliminated. Another finding was that if the SCP-containing compound was less than 5% (v/v), the coalescence between droplets became severe (Fig. 3a and b, condition 14). We identified a zone in the 3-component phase diagram (the light-blue region in Fig. 3a) within which the droplets generated by MiCA centrifugation were monodispersed (coefficient of variation (CV) ≤ 0.05, typically) with moderate thermal stability to survive more than 24 h at room temperature and no more than 30 thermal cycles of PCR (ESI† Fig. S3). The oil mixture within this range may be used in experiments without strict requirements for thermal stability, particularly isothermal amplification methods such as emulsion LAMP or emulsion MDA.

Although the absence of mineral oil leads to better droplet uniformity throughout the thermal cycling process, the thermal stability of such binary mixtures of DEC and SCP-containing compounds is not ideal and slight coalescence is still apparent in the emulsion after many thermal cycles (Fig. 3c). An emulsion is a nonequilibrium system with a tendency to be thermodynamically demulsified into separated phases. To further facilitate the thermal stability, we explored similar silicone materials and low viscosity oil compounds. The SCP-containing compound we used was Abil WE09, a mixture of polyglyceryl-4 isostearate, cetyl PEG/PPG-10/1 dimethicone, and hexyl laurate, which as a whole has an HLB value of approximately 5. We found that Abil EM90 and EM180, which contain only cetyl PEG/PPG-10/1 dimethicone and no other esters, also had an HLB value of around 5, which is appropriate for our applications. In addition, we found that isopropyl palmitate has a similar viscosity to DEC (*ca.* 6 cSt at 25 °C), making it another possible substitute for DEC. We then compared the thermal stability of the emulsions using different combinations of these materials and increased the number of PCR cycles to 40. With 93% (v/v) isopropyl palmitate supplemented with 7% Abil EM180, the formula exhibited good compatibility in the MiCA system, producing monodisperse droplets with good thermal stability to survive 40 thermo-cycles of PCR (Fig. 3c). Our result suggests that isopropyl palmitate is more compatible with cetyl PEG/PPG-10/1 dimethicone. The exact reason for this observation is still unclear. One of the possible speculations is that the one long carbon chain may give them better affinity. We may need more

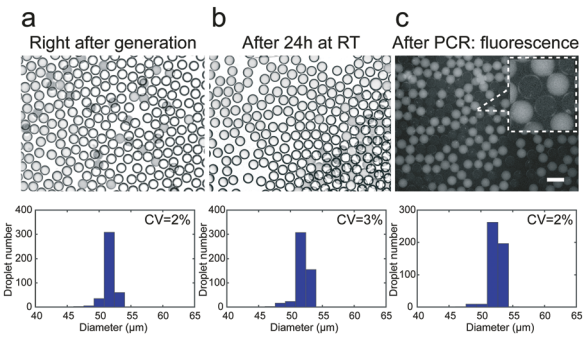

Fig. 4 Droplets generated with the digital PCR reaction mixture and an oil mixture of 93% (v/v) isopropyl palmitate supplemented with 7% (v/v) Abil EM180. The droplets remained monodisperse and thermally stable from initial generation (a), to 24 h room temperature resting (b) and after 40 cycles of thermal ramping (c). Within the droplets, digital amplification took place with the fluorescence signals being well segregated. The droplets showed little shrinkage or expansion. Scale bar: 100 μm.

evidence using other long carbon chain compounds to understand the mechanism better in the future.

We then used this binary formula to perform digital PCR experiments where the droplets exhibited satisfactory thermal stability and coalescence resistance (Fig. 4). We used a 280 bp double-stranded DNA fragment from the prfA gene of *L. monocytogenes* as the template and detected a specific region with a TaqMan probe. 20 μL of reaction mixture was prepared and transferred to the upper surface of a MiCA plate in an assembled MiCA emulsifier, which was then put into a microcentrifuge tube with the oil phase preloaded. After several minutes of centrifugation at 13 000*g*, the aqueous solution was completely transformed into uniform emulsion droplets with a mean diameter of 52 μm (CV = 2%, Fig. 4a). The generated emulsion was stable at room temperature for over 24 h (droplet mean diameter = 52 μm, CV = 3%, Fig. 4b). After 40 thermal cycles of PCR, the compartmented target template DNAs were amplified and the fluorophores of the TaqMan probes were activated, making the droplets fluorescent (droplet mean diameter = 53 μm, CV = 2%, Fig. 4c and ESI† S4). By counting the number of positive droplets, the specific amount of the original template could be calculated according to the Poisson distribution. The emulsion droplets generated with an oil mixture of 93% (v/v) isopropyl palmitate supplemented with 7% (v/v) SCP (Abil EM180) were highly uniform and stable at room temperature and throughout all of the thermal cycles, which also validated the high performance of the binary formula.

Although we have seen a trend in the survey diagram, we still lack a solid standard to draw a clear-cut line of the boundary and we blurred the area that we have less confidence in. This is to leave enough room for adjustment in search for a satisfactory formulation. Emulsion stability remains an extremely challenging issue to address, and many second-order effects in this complex nonequilibrium system can affect the result. There seems to be no easy way of predicting a perfect or suitable formulation for a given case, and experimental validation is always needed.

Conclusions

Compartmentalized PCR has demonstrated its utility in quantitation and amplification purposes and is still being explored for new applications where emulsion-based PCR plays a significant role. Using a centrifugation-driven MiCA emulsion generator, the emulsification process enjoys faster speeds and a much higher throughput without complex control systems and tedious handwork. Since a stable emulsion is a prerequisite in emulsion PCR, a suitable oil–surfactant mixture assures the application of MiCA. In this work, we first tested prevalent oil–surfactant mixtures used in emulsion PCR and by systematic searching, we found a formulation that outperforms its predecessors. We first found that diethylhexyl carbonate (DEC) with 7% (v/v) silicone copolymer (SCP)-containing compound (Abil WE09) could generate monodisperse droplets; however, the thermal stability was not ideal for PCR thermal cycling. We therefore looked for low viscosity oils and water-soluble surfactants with better oil affinity and developed a new formula; isopropyl palmitate with 7% (v/v) SCP (Abil EM180), which was shown to perfectly fit the MiCA droplet generation protocol and shows excellent thermal stability for use in digital PCR. We believe that this new formula, as well as the systematic survey of the mechanical and thermal stability of the emulsions with different emulsion oil combinations, provides a detailed landscape for understanding oil-phase formulation to support emulsion DNA amplification and achieving better performance in amplification reactions.

Author contributions

Y. H. conceived the idea and designed the study. F. Z., P. L., Y. S. and Z. C. conducted the experiments. F. Z., P. L., Z. C., Y. P. and Y. H. analysed the data. F. Z., P. L and Y. H. wrote the paper. All authors commented on the manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

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