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Influence of segmenting fluids on efficiency, crossing point and fluorescence level in real time quantitative PCR

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Abstract The two-phase segmented flow approach to the processing and quantitative analysis of biological samples in microdevices offers significant advantages over the single-phase continuous flow methodology. Despite this, little is known about the compatibility of samples and reactants with segmenting fluids, although a number of investigators have reported reduced yield and inhibition of enzymatic reactions depending on the segmenting fluid employed. The current study addresses the compatibility of various segmenting fluids with real time quantitative PCR to understand the physicochemical requirements of this important reaction in biotechnology. The results demonstrate that creating a static segmenting fluid/PCR mix interface has a negligible impact on the reaction efficiency, crossing threshold and end fluorescence levels using a variety of segmenting fluids. The implication is then that the previously reported inhibitory effects are the result of the dynamic motion between the segmenting fluid and the sample in continuously flowing systems. The results presented here are a first step towards understanding the limitations of the segmented flow methodology, which are necessary to bring this approach into mainstream use.

Keywords Polymerase chain reaction · Microfluidic analytical techniques · Biocompatible materials

Introduction

To date, the use of lab on a chip technology presents a number of advantages in a broad range of analytical techniques and applications. In the case of biological samples, examples include electrophoresis (Austin, 1993; Woolley, 1994) and the polymerase chain reaction (PCR) (Leamon, 2003; Nagai, 2001a, 2001b; Chaudhari, 1998). As the focus in analytical chemistry progressed to the micro scale in recent years, surface effects took on a new level of importance, equaling that of biology (Yang, 2002). Reduced reaction efficiency and inhibition in microdevices has been attributed to the increased interface surface to sample volume ratio (Panaro, 2004; Shoffner, 1996). Hence, a new interest in materials compatibility emerged, as reflected in the proliferation of multidisciplinary research. The almost exclusive aim of this research was the development of an ideal material suitable for biological analysis in lab-on-a-chip microdevices. Substrate materials used to fabricate devices include glass, borofloat-glass (Ferrance, 2003), fused silica (Song, 2004; Huang, 2003), silica (Kirby, 2003), soda-lime glass, silicon (Zhao, 2003; Gabig-Ciminska, 2004), SU-8, Acrylic (Huang, 2003), PDMS (Marquette, 2004; Xiao, 2004), PEEK (Park, 2003), COC, PMMA (Lee, 2004), Polyimide (Huang, 2003), PTFE, fluorocarbon film, PC (Liu, 2004), diamond coated chip (Hanaoka, 2003) and zeonor (Lai, 2004). Despite these concentrated efforts, the search for the perfect material for TAS applications has, thus far, proved elusive. Another approach to circumvent the problem has been to passivate the material surfaces that come in contact with the reaction components to minimize adverse effects on the reaction outcome. Again, a number of different techniques have been proposed, the most popular being silanisation, which is believed to reduce the adsorption of macromolecules. Other surface treatments include a mixture of polyethylene glycol and polyacrylic, polyacrylamide gel (Xiao, 2004), parylene (Shin, 2003), silicone oxide and silicone nitride (Shoffner, 1996). However, these processes are time-consuming and, more worryingly, criticism regarding

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their reproducibility has been raised, questioning the entire methodology.

A relatively new and exciting approach to the processing of massive numbers of samples efficiently in serial format is the use of continuous two-phase segmented flows, which has received much attention in the recent literature for the processing of samples in microdevices (Auroux, 2004; Burns, 2001; Song, 2003; Nisisako, 2002; Grodrian, 2004; Köhler, 2004; Schneegass, 2001; Henkel, 2004; Curcio 2003; Park, 2003; Obeid, 2003). This approach, where samples are separated to form either slugs or droplets by a segmenting fluid, presents great advantages over the single phase methodology, including reduced cross-contamination, reusability of the device, absence of contact between solid surfaces and sample, and easier control of samples. In a special report dedicated to the science and application of segmented fluids in microfluidic devices, the recent growth in this area was acknowledged and the current and future possibilities highlighted (Jensen, 2004). This approach has proved useful for cell culture and other miniaturized chemical and biological operations, including PCR in carrier fluids such as perfluorodecalin (Curcio, 2003), air (Park, 2003; Obeid, 2003), fluorinated oil FC-40 combined with fluoroalcohol surfactants (Dorfman, 2005), dodecane (Schneegass, 2001), mineral oil (Schneegass, 2001), and hydrocarbon octane. However, variations in product yield were reported depending on the fluids used (Köhler, 2004; Dorfman, 2005), the inconsistent results being attributed to the influence of the segmenting fluid on the PCR. In other studies, using emulsified microdroplets in oil as PCR reactors for the amplification of a single molecule (Nakano, 2004), or with a number of fluids combined with Span80 (Tawfik, 1998) and Tween80 (Song, 2000) surfactants, generated results ranging from no yield to high efficiency. It has been suggested that a reason for the inhibition of enzymatic reactions may lie in the aggregation of macromolecules at the oil/aqueous interface (Lopez-Quintela, 2004). It has also been demonstrated recently that in both slug and droplet segmented flows, the sample may never come in contact with a solid surface when an appropriate segmenting fluid is utilized because carry over between samples could not be detected (Dorfman, 2005). This has massive implications for the segmenting fluid approach, overtaking the importance of material surface and material treatments compatibility mentioned earlier.

Thus, the need for further investigation into fluid compatibility is clear. The importance of this transformation into multiphase systems is further highlighted by recent articles addressing the engineering physics and additional control possibilities for microscale chemical reactions (Link, 2004; Cristine, 2004). However, detailed understanding based on experimental evidence is crucial for the development of two-phase segmented flow PCR. Little work has been undertaken to systematically assess the effect of segmenting fluids in biological reactions of interest for biomedical microdevices. The present study will focus upon the compatibility of various segmenting fluids with PCR. As the most sensitive analytical technique for genetic analysis, the compatibility of segmenting fluids can be optimally evaluated, as minute differences may be amplified to detectable levels. Also, PCR is currently well understood and it provides an excellent reference standard for the development of compatible fluids. Properties immediately determinable from PCR fluorescence curves include reaction efficiency, minimum detectable amplicon levels and total fluorescence levels, all of which can be evaluated with variations in constituents of original PCR mix or the number of starting DNA template copies. These tests are the first to examine the compatibility of segmenting fluids on quantitative PCR parameters such as efficiency, estimated crossing point and end fluorescence intensity. Such an investigation is a crucial first step for the future development of the segmented flow approach to lab on a chip technology.

Experimental methods

PCR reaction

The PCR reaction for each experiment consisted of LightCycler FastStart DNA Master SYBR Green I (Roche, 3 003 230). A 240 bp fragment of the pGEM®-5Zf(+) vector was amplified using the following primers: M13 uni (−43) 5′-AGG GTT TTC CCA GTC ACG ACG TT-3′ and M13 rev (−29) 5′-CAG GAA ACA GCT ATG ACC-3′. The 240 bp fragment was initially isolated by PCR and purified using a QIAquick PCR Purification Kit (Qiagen, Cat No. 28104).

The intercalating dye SYBR Green I was chosen as the detection method as it closely follows the logistic shape of typical PCR S-curves. PCR was performed using a LightCycler (Roche Diagnostics) according to the following protocol: 10 minute initial denaturation at 95°C followed by 30 cycles consisting of denaturation at 95°C for 5 seconds, annealing at 55°C for 5 seconds and extension at 72°C for 10 seconds. Ramp rates between each temperature were set to 20°C/s. Fluorescence was monitored at the end of each cycle using the F1 channel, excitation wavelength 470 nm, emission wavelength 530 nm. After the 30 cycles of amplification a melt curve was obtained using the following thermal profile: transition from 65°C to 95°C at 0.1°C/s with continuous monitoring of fluorescence. A final cooling step was performed, bringing the samples down to 40°C to finish the thermal cycle.

Evaluation of fluids

The following fluids were chosen for evaluation of their compatibility with the PCR mixture as they have been

quoted in the literature as segmenting fluids (Tawfik, 1998; Wittwer, 1997). The fluids evaluated are: Fluorinert FC-40 (Sigma-Aldrich, F9755); N-Dodecane Oekanal (Riedel-Sigma-Aldrich 46155); Dodecane (Fluka-Sigma-Aldrich, 44030); Tetradecane 99+% (Aldrich-Sigma-Aldrich, 172456) and N-Tetradecane Oekanal (Riedel-Sigma-Aldrich, 46180). Two grades of Dodecane and Tetradecane were used to establish if this was the cause of the reported variations in specific product yield.

The procedure followed to evaluate each fluid was as follows. A PCR master mix was made up in accordance with specifications supplied with the LightCycler FastStart DNA Master SYBR Green I kit. A no template control sample (NTC) was pipetted into the LightCycler capillary before the addition of template DNA. The template DNA sample (concentration 10 pg/μl) was then added to the master mix and pipetted into two separate capillaries as positive controls. One positive control was left in the cooling block while the other positive control was wrapped in tinfoil and placed in a fridge at 4°C. Thirty μl of PCR mixture was pipetted into each of six 0.5 ml eppendorfs. Four-hunderd μl of the test fluid was subsequently layered and left in contact with the PCR mix. Three samples remained in contact for 15 minutes while the remaining three samples remained in contact for 60 minutes. The eppendorfs were left in the cool block for the duration of the experiment. For all carrier fluids except for Fluorinert FC-40, the PCR mix remained at the bottom of the eppendorf, with the carrier fluid covering it, i.e. the density of the PCR mix was greater than the segmenting fluid. While in contact with the FC-40, the PCR mixture formed a floating globule on top of the FC-40, i.e. the density of the PCR mix was less than the segmenting fluid. After the respective times had elapsed, 20μl of PCR mixture was removed from each eppendorf and pipetted into individual capillaries which were then capped. All capillaries were centrifuged for 5 seconds at 700 g to ensure that the PCR mixture filled the bottom of the capillary and none of the potential segmenting fluid was present. The capillaries were then loaded into the LightCycler and underwent amplification according to the previously described protocol.

Standards were prepared separately. Eighteen μl of freshly prepared PCR mix was pipetted into each of five capillaries and 2 μl of DNA (concentrations 10, 1, 0.1 and 0.01 pg/μl) were added to the first four capillaries respectively, while 2 μl of sterile water was added to the last capillary. The capillaries were then capped, centrifuged and loaded into the LightCycler for amplification and melt curve analysis to check that the specific product had been amplified. In all cases a single peak in the first derivative of the fluorescence curve was found to occur within a temperature range of less than 1 degree demonstrating a yield of specific product.

Data analysis methods

The results of the PCR amplification have been analysed in terms of PCR efficiency, crossing threshold and end fluorescence intensity. The efficiency of the reactions was first calculated using the standard curve method. This method calculates the average efficiency for a set of standard curves and assumes that the efficiency of the test reactions performed is the same as the average standard efficiency. The efficiency value is obtained by plotting the crossing threshold against the log of DNA concentration. The PCR efficiency is then calculated from the slope of the standard curve (Efficiency $= 10^{-1/\text{slope}}$).

The Ramakers method (Ramakers, 2003) was also used to calculate the efficiency of each individual reaction. The LinRegPCR software version 7.5, (available at request from bioinfo@amc.uva.nl Subject: LinRegPCR), was used to extract this data. This method allowed the comparison of efficiencies between individual samples and the standards, which were not in contact with the segmenting fluid. It is based upon taking the best linear fit of the PCR fluorescence curve linear region and can be applied to each individual sample. The efficiency (Eff) can be calculated by obtaining the slope of the linear section of a curve of Log(Fluorescence) versus cycle number and substituting the slope into the equation Eff $= 10^{\text{slope}}$.

The crossing threshold was taken from the LightCycler data analysis software using the second derivative maximum method with an arithmetic baseline adjustment in accordance with recommendations for performing experiments using SYBR Green I. The crossing threshold is the cycle number at which the fluorescence of each sample is the same value just above the background fluorescence (Wittwer, 1997) and is indicative of the starting of detectable amplification of the DNA template. Finally, the end fluorescence level was recorded and compared with the control value.

Comparison of parameters across the different fluids were done using ANOVA.

Results and discussion

Figures 1 to 3 provide the first insight of the relative compatibility of PCR with a number of potential segmenting fluids. Figure 1 shows the estimated efficiency values obtained in the presence of the different segmenting fluids. The PCR efficiency of the standards (labelled Eff (Std)) was calculated from the slope of the standard curve. Additionally, the Ramakers method to calculate efficiency was applied to each individual standard and averaged to give the presented data (labelled Eff (Ram) Std). Both methods showed good agreement. Therefore, the Ramakers method was subsequently

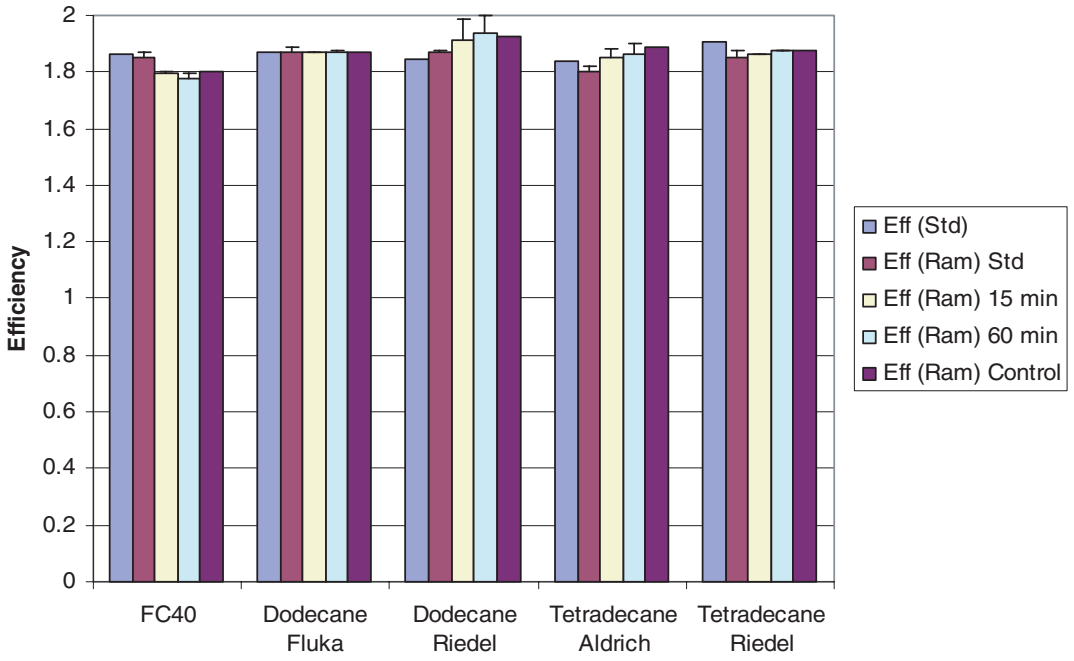

Fig. 1 Efficiency obtained from dilution standards using the Log fluorescence relationship method and from the Ramakers methods for each individual sample. The triplicate samples were averaged in the Ramakers method to obtain the plotted value

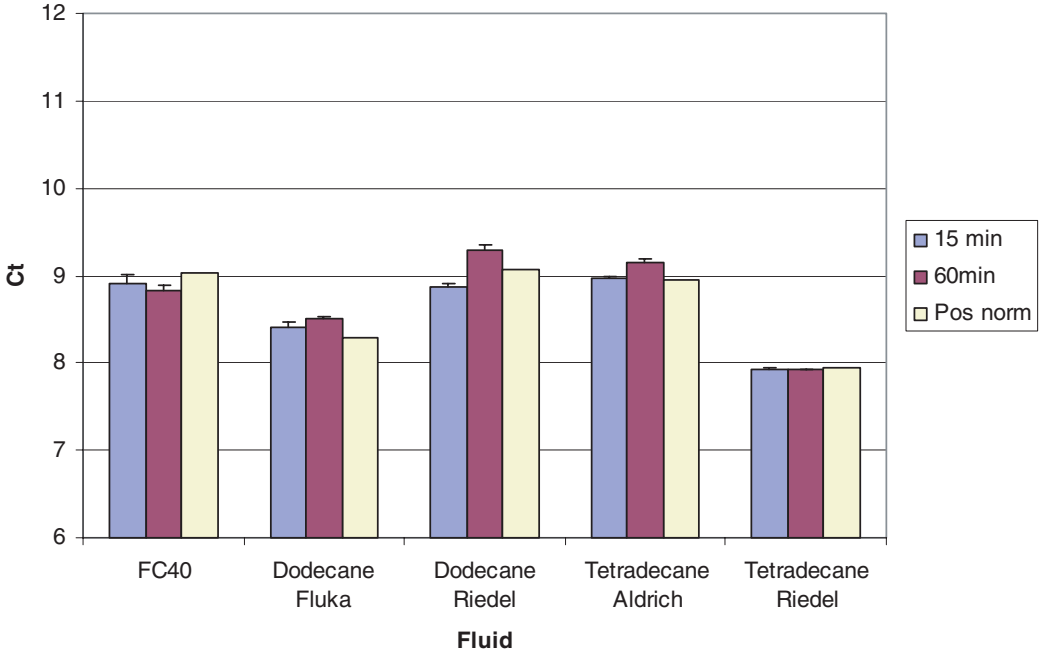

Fig. 2 Estimated crossing threshold (Ct) obtained from the quantitative real time PCR amplification curves of a DNA sample (20 pg) exposed to segmenting fluids for 15, 60 minutes and compared to the same sample not exposed (control). Each value represents an average of the triplicate sample set tested

applied to each sample under investigation, with the average values being presented from triplicate tests. Clearly, the efficiency appears to be unaltered by creating the segmenting fluid/PCR mix static interface prior to amplification. This indicates that the segmenting fluids do not adversely affect PCR efficiency, as several studies suggest. This is based on the observed partial inhibition of catalytic activity of enzymes put into contact with hydrophobic solvents in biphasic fluidic systems (Lopez-Quintela, 2004). Interaction of hydrophobic domains of the enzyme with hydrophobic interfaces can lead to aggregation and loss of activity, impairing reaction yield and efficiency. This is especially relevant in microfluidic

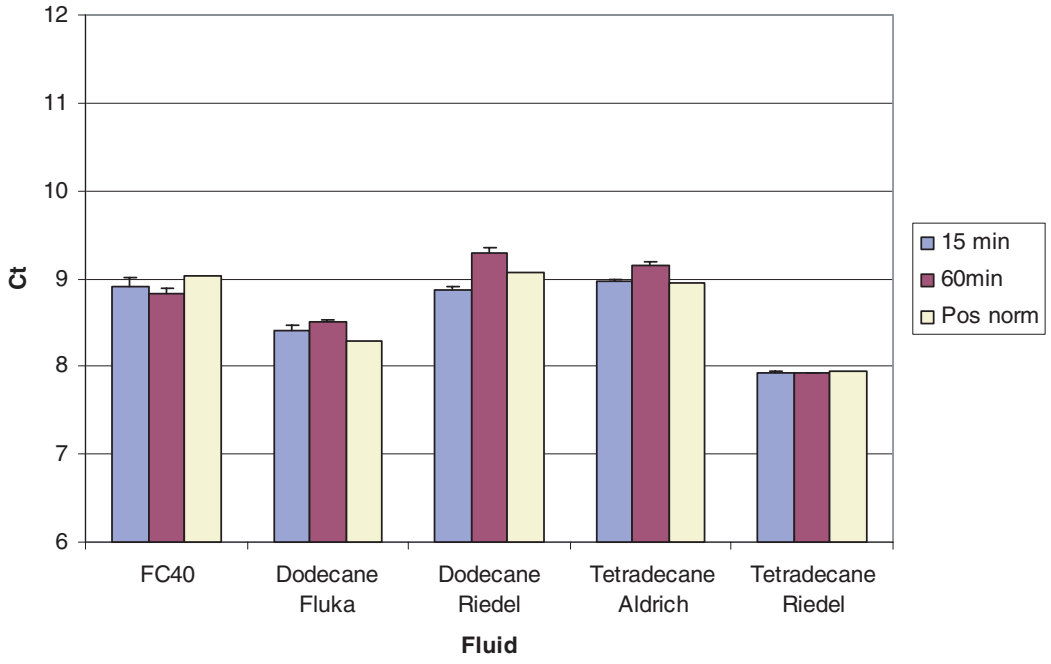

Fig. 3 End fluorescence values obtained from amplification curves of a DNA sample (20 pg) in contact with different segmenting fluids and normalized upon the control (not exposed). Each value represents an average of the triplicate sample set tested

devices, where the surface to volume ratio is greatly enhanced. We can rule out this possibility in the case of PCR and the tested fluids.

An additional test for fluid compatibility is to study its influence on the estimated crossing threshold for a particular biological sample. The crossing threshold (Ct) is the critical parameter used in quantitative real time PCR to calculate nucleic acid concentration in samples. Figure 2 shows that the crossing threshold level is unaffected by the presence of the segmenting fluid prior to amplification. The length of time spent in contact with the segmenting fluid has no effect, nor has the commercial source of the fluid. These results indicate that the estimation of the concentration of a DNA template sample is unaffected by the segmenting fluid/PCR mix interface. This implies that no diffusion of PCR components takes place at the segmenting fluid/PCR interface prior to amplification in these fluids. This result is especially important in serial continuous-flow devices, as diffusion from the sample into the segmenting fluid may result not only in misleading calculations, but also in cross-contamination inside the serial sample train, placing questions over the segmented flow methodology.

Finally, we analyze the PCR curves to inquire if the fluids may have a negative effect on the measurement of the fluorescence. This question cannot be inferred from the previous analyses. The rationale behind it is that, even with no detrimental effect of the fluids on the PCR itself, SYBR Green I may still escape into the segmenting fluid, therefore affecting the detectability capability of the analytical system. Again, the end fluorescence levels are largely unaffected by the presence of the segmenting fluid, relative to the control samples. This is an important and promising result as most of the detection techniques under development for PCR microdevices base their signal upon SYBR Green I fluorescence or similar fluorochromes. Any interference in the end fluorescence levels would cause design problems for the optical detection of amplicons in real time PCR.

From these experiments, we conclude that the chosen segmenting fluids have no intrinsic effect on PCR efficiency or detectability. This contrasts with reports from the literature where it has been found that using dodecane as a segmenting fluid results in low product amount (Schneegass, 2001) and that the fluid FC-40 required the addition of surfactants to achieve good amplification results (Dorfman, 2005). The current work demonstrates that the inhibitory effects are not caused by the creation of the segmenting fluid/PCR interface. The reasons for the drop in product yield in these studies must be caused by other properties of the segmenting fluid as it flows, enhancing chemical, thermal and mechanical deleterious effects. For example, vortices can form within flowing slugs or droplets, which will amplify any inhibitory effects due to the segmenting fluid/PCR interface. Additionally, slug/droplet stability may not be achieved without the addition of surfactant to the PCR mix or segmenting fluid, hence the sample may come in contact with solid material surfaces, which are known to have a negative impact on PCR yield. Although much work remains to be done, the current paper provides a good basis for ruling out potential inhibitory mechanism in segmented flows, hence increasing the understanding of the approach.

Conclusions

The first compatibility study of segmenting fluids with real-time quantitative PCR efficiency, crossing threshold and fluorescence development was conducted. The experiments have demonstrated that creating a static segmenting fluid/PCR mix interface prior to amplification has little if any effect on key PCR parameters. This is a favourable result for the future of the segmented flow approach. We acknowledge the limitations of our experimental approach. Thus, we intentionally neglected addressing the effects of dynamic flowing samples, which would be a necessary step to understanding the origins of the inhibition reported in the literature. However, this work is a significant initial step in the development of a rational segmented flow approach which will help in the understanding of inhibition mechanisms at the microscale. Systematic studies such as the one presented here will be necessary, as the compatibility of the fluid-to-fluid interface must be understood to achieve reliable fluid design for PCR microfluidic devices.

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