Using Three-Phase Flow of Immiscible Liquids To Prevent Coalescence of Droplets in Microfluidic Channels: Criteria To Identify the Third Liquid and Validation with Protein Crystallization

Delai L. Chen, Liang Li, Sebastian Reyes, David N. Adamson, and Rustem F. Ismagilov*

Department of Chemistry and Institute for Biophysical Dynamics, The University of Chicago, 929 East 57th Street, Chicago, Illinois 60637

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This manuscript describes the effect of interfacial tensions on three-phase liquid−liquid−liquid flow in microfluidic channels and the use of this flow to prevent microfluidic plugs from coalescing. One problem in using microfluidic plugs as microreactors is the coalescence of adjacent plugs caused by the relative motion of plugs during flow. Here, coalescence of reagent plugs was eliminated by using plugs of a third immiscible liquid as spacers to separate adjacent reagent plugs. This work tested the requirements of interfacial tensions for plugs of a third liquid to be effective spacers. Two candidates satisfying the requirements were identified, and one of these liquids was used in the crystallization of protein human Tdp1 to demonstrate its compatibility with protein crystallization in plugs. This method for identifying immiscible liquids for use as a spacer will also be useful for applications involving manipulation of large arrays of droplets in microfluidic channels.

Introduction

This paper describes a method to prevent the coalescence of adjacent reagent plugs in plug-based microfluidics.[1] Discrete microfluidic plugs[1−3] (droplets large enough to fill the cross section of a microfluidic channel) dispersed in an immiscible carrier fluid have been used in protein crystallization,[4−6] synthesis of microparticles[7] (including vesicles[8,9] and capsules[10,11]) and double emulsions,[12,13] enzymatic assays,[6] protein expression,[14] and screening reaction conditions.[15] The coalescence of neighboring plugs (Figure 1a), however, can cause contamination of reagents, change in the size of plugs, and make it difficult to locate an individual plug within a sequence of plugs. Coalescence is driven by interfacial energy and can occur when two plugs of the same phase catch up and come into contact as a result of the relative motion of plugs during flow. Relative motion is more likely for adjacent plugs containing solutions of different viscosities or interfacial tensions.[16,17] Even for plugs containing the same solution, relative motion may take place if the sizes of adjacent plugs are different, a phenomenon that was previously used to direct the coalescence of plugs.[1] Coalescence may be suppressed by loading the liquid−liquid interfaces with detergents[18] or colloidal particles,[19,20] but this manipulation of interfaces may be undesirable for some applications. For example, some detergents cause proteins to adsorb to the fluid interface.[21,22] To avoid these potential problems, we have chosen to eliminate coalescence by preventing direct contact of adjacent reagent plugs.

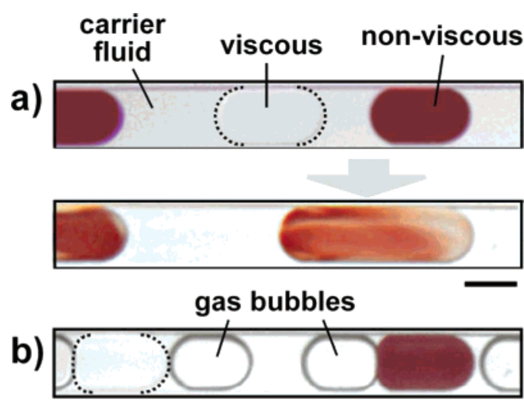

Figure 1. (a) Plugs of two reagents (white and red) having different viscosities coalesced during flow. (b) Gas bubbles were previously used as spacers to prevent the red and white plugs from coalescing. These microphotographs were previously published in ref 29. Scale bars are 800 μm.

* Corresponding author. E-mail: r-ismagilov@uchicago.edu.

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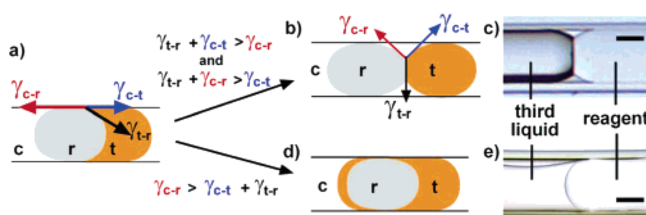


Figure 2. Predicting engulfing of plugs by analyzing the interfacial tensions. (a) A hypothetical starting position in which the third liquid (t) and the reagent plug (r) are in complete contact. This situation may be unstable because the interfacial forces (represented by γ, which is the interfacial force per unit length of the contact line) are not balanced. Abbreviations: c, carrier fluid; r, reagent; t, third liquid. (b) The interfacial forces equilibrate, and engulfing does not occur for high γ_{t-r}. (c) A microphotograph of a third liquid plug separating reagent plugs. Third liquid: SID; reagent: 15% glycerol; carrier fluid: FC3283/PFO (10:1, v:v). (d) The third liquid plug completely engulfs the reagent plug for low γ_{t-r}. (e) A microphotograph of the third liquid engulfing a reagent plug. Third liquid: DTFS; reagent: water; carrier fluid: FC40. The scale bars in (c) and (e) are 100 μm.

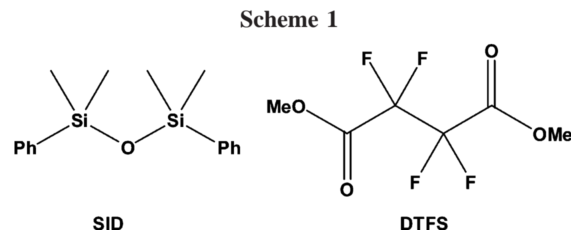
We have previously used gas bubbles[6,15,17] (Figure 1b) to separate reagent plugs, resulting in a three-phase flow of gas−reagent−carrier.[23,24] Gas bubbles were used in liquid−gas two-phase segmented flow as well.[25,26] For applications involving long arrays of plugs, two drawbacks may need to be overcome in using gas bubbles as spacers: (1) compressible gas bubbles could cause flow fluctuation and a lag in response to the change of flow rates in pressure-driven flow, and (2) gas bubbles may dissolve in the fluorinated carrier fluid under high pressure.[27] Solving these problems would be especially useful when performing screens using cartridges[28] preloaded with reagent plugs. In these screens, a stream of a substrate solution is injected into plugs in a preformed array through a T-junction, with each plug containing a solution of a different composition.[6,15,28]

Here, we used plugs of a third immiscible liquid, instead of gas bubbles, as spacers to prevent coalescence of adjacent reagent plugs. In the following discussion, "third liquid" refers to any liquid immiscible with the carrier fluid and the reagent, while "spacers" refers to third liquids that are effective in preventing coalescence. We tested the requirements of interfacial tensions for plugs of a third liquid to act as spacers, identified two candidates satisfying the requirements, and demonstrated the compatibility of one of these liquids with protein crystallization.

Experimental Section

Materials. The glycerol solutions were made in water, and the percentage concentrations were by volume unless otherwise stated. The three carrier fluids were fluorocarbons used with or without the surfactant 1,1,2,2-tetrahydroperfluorooctanol (PFO), provided by Alfa Aesar, MA: (1) FC40, provided by Acros Organics, NJ; (2) FC70; and (3) FC3283, both provided by 3M, MN. 1,3-Diphenyl-1,1,3,3-tetramethyldisiloxane (SID, Scheme 1) was purchased from Gelest, PA. Dimethyl tetrafluorosuccinate (DTFS, Scheme 1) was obtained from Synquest, FL. Protein Tdp1 (N-terminal truncation (Δ1-148) of the human tyrosyl-DNA phosphodiesterase with an N-terminal His-tag, expressed in *Escherichia coli*) was provided by

Scheme 1



deCODE biostructures, WA. The protein solution was provided frozen, at a concentration of ~6.7 mg/mL in a buffer containing 250 mM NaCl, 15 mM Tris (pH 8.2), and 2 mM Tris(2-carboxyethyl)-phosphine (TCEP). A detailed description of the protein expression and purification can be found in ref 30. *N*,*N*-Dimethyldodecylamine *N*-oxide (LDAO) was purchased from Fluka, Switzerland.

Measuring Interfacial Tensions. Interfacial tensions were measured using the pendent drop method on Advanced Digital Automated Goniometer, model 500, from Ramé-Hart Instrument, NJ, with data analysis by software DROPimage Advanced version 1.5.04. To obtain the equilibrium interfacial tensions in the three-phase system of FC40−LDAO−DTFS, the three phases were first pre-equilibrated by combining and extensively mixing equal volumes of each phase in a vial before interfacial tension measurement. To obtain the equilibrium interfacial tensions in the three-phase system of FC3283−LDAO−SID, the three phases were pre-equilibrated by combining and keeping the three phases in a vial for 24 h with only occasional gentle shaking (to prevent the formation of a stable emulsion). Interfacial tensions were then measured between every two phases.

Visualizing the Interface between the Third Liquid and the Reagent Plug. An array of alternating third liquid and reagent plugs was formed by aspirating the third liquid, the carrier fluid, and the reagent solution into a piece of Teflon tubing (200-μm i.d.) pre-filled with carrier fluid.[6] To visualize the third liquid−reagent interface, plugs were manually driven back and forth using a syringe connected to the tubing until the third liquid and the reagent plug came into contact. Microphotographs of the interfaces were taken using a Leica MZ 12.5 stereoscope equipped with a Spot Insight color digital camera (model 3.2.0).

Separating Plugs of Different Viscosities with Plugs of the Third Liquid. PDMS microfluidic devices with channels of square cross sections (200 × 200 μm^2) were fabricated by rapid-prototyping soft lithography.[31,32] Alternating plugs[33] of a viscous solution (30% glycerol) and a nonviscous aqueous solution (a mixture of 0.07 M $Fe(SCN)_3$ and 0.21 M KNO_3) were generated in a microfluidic device using FC70/PFO (10:1, v:v) as the carrier fluid (Figure 3a). A stream of the third liquid was introduced downstream of the point of alternating plug formation so that a plug of the third liquid was inserted between every pair of viscous and nonviscous plugs. Teflon tubing (200-μm i.d.) was connected to the outlet of the PDMS channel to extend the flow path. Microphotographs of the droplets were taken at different points along the flow path. To test if the plugs coalesced without the third liquid, a control experiment was performed without the stream of the third liquid.

Injecting a Substrate Stream into Reagent Plugs Separated by Plugs of the Third Liquid. A T-junction microfluidic device (Figure 4a) was fabricated from a piece of PDMS imprinted with the channel features and a glass slide. The PDMS piece and the glass slide were first plasma oxidized and then sealed together to form the channels. The channel surface was rendered hydrophobic by silanization as described previously.[21] A hydrophilic glass capillary was inserted from the vertical branch to the junction point of the T-junction and used to inject substrate solution. The horizontal branches of the T-junction were connected to Teflon tubing. An

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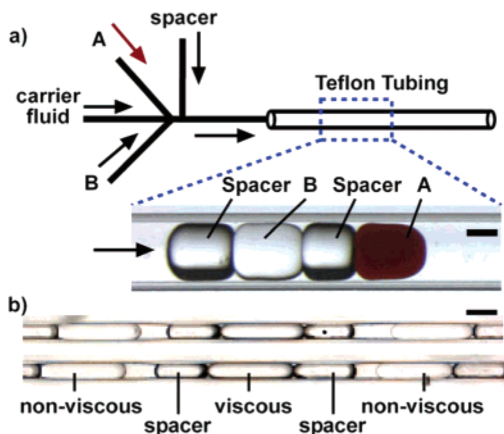

Figure 3. Separating alternating plugs containing solutions of different viscosities with SID plugs. (a) Above: A schematic of the microfluidic device used. The carrier fluid was FC70/PFO (10:1, v:v); the spacer was SID; A was 0.07 M $Fe(SCN)_3$ and 0.21 M KNO_3; and B was 30% glycerol. Below: A microphotograph of the plugs flowing in the Teflon tubing. Flow rates: carrier fluid, 4 μL/min; A and B, 2 μL/min each; spacer, 2 μL/min. The scale bar is 100 μm. (b) Microphotographs of plugs in two side-by-side PDMS channels resulting from splitting an array of larger plugs. The channels had a square cross section of 200 × 200 μm^2. Viscous solution: 70% glycerol. Nonviscous solution: water. Carrier fluid: FC3283/PFO (10:1, v:v). The scale bar is 320 μm.

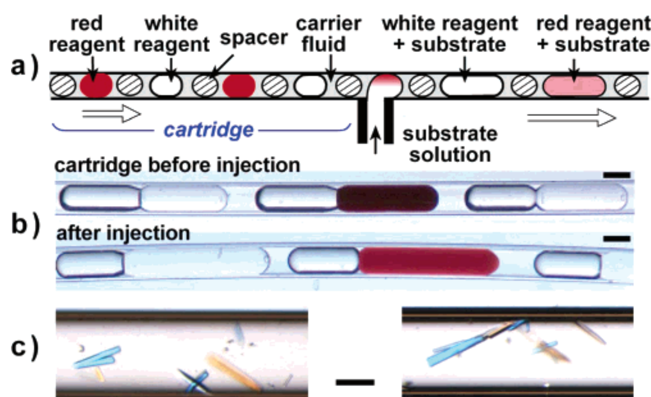

Figure 4. (a) A schematic of the T-junction microfluidic device used for injecting plugs from a preloaded cartridge with a substrate solution. The plugs were 30% aqueous glycerol (colorless) and an aqueous solution of 0.07 M $Fe(SCN)_3$ (red) separated with SID plugs. (b) Microphotographs of the cartridge before (top) and after injection (bottom) with a colorless solution. Flow rates: substrate, 0.4 μL/min; plugs, 1.0 μL/min. Scale bars are 200 μm. (c) Protein Tdp1 crystallized in the presence of SID plugs. The scale bar is 100 μm.

array of alternating plugs of viscous and nonviscous solutions separated by plugs of the third liquid was driven through the Teflon tubing connected to the horizontal branch of the T-junction, and the substrate was injected from the glass capillary. The resulting plugs were collected in the Teflon tubing connected to the downstream horizontal branch of the T-junction. The carrier fluid was FC3283/PFO (10:1, v:v). Water was used in this experiment to mimic the substrate solution.

Crystallizing Tdp1 in the Presence of SID. Alternating plugs of the precipitant (22% PEG-3000, 0.2 M NH_4Ac, 0.1 M HEPES buffered at pH 7.5) for Tdp1 and plugs of SID were aspirated,[6] using FC3283/PFO (10:1, v:v) as the carrier fluid. The plugs were injected with a stream of Tdp1 solution using the same method described in the previous section. The resulting plugs of crystallization trials and plugs of the third liquid were flowed into a silanized glass capillary.[34] The capillary was sealed and incubated at 23 °C and checked every 2 days for crystal formation. Protein crystals were observed in about 50% of the plugs on the fourth day of incubation.

Results and Discussion

We first tested the conditions under which the third liquid can effectively prevent direct contact between reagent plugs. We found that when a plug of the third liquid was brought in contact with a reagent plug (Figure 2a), the third liquid might form a plug clearly distinguishable from the reagent plug (Figure 2b,c), or the third liquid might "engulf" the reagent plug (coat the reagent plug without coalescing) (Figure 2d,e). In the case of engulfing, we observed that the third liquid may transfer from one end of the reagent plug to the other during flow and not effectively prevent the direct contact or coalescence of reagent plugs. Therefore, we focused on preventing the plug of third liquid from engulfing the reagent plug.

To understand the factors affecting engulfing, we have made two assumptions. First, the carrier fluid preferentially wets the channel, so that plugs of the third liquid and plugs of the reagent are surrounded by a thin film of the carrier fluid and do not touch the channel.[35] This assumption will ensure that plugs of the third liquid and reagent can be formed. Second, the capillary number (*Ca*)[36] is small. *Ca* relates viscous forces to interfacial forces: $Ca = \eta U/\gamma$, where η [kg m^{-1} s^{-1}] is the viscosity, U [m s^{-1}] is the flow velocity, and γ [N m^{-1}] is the interfacial tension. This assumption assures that viscous forces are negligible compared to interfacial forces and that engulfing is dominated only by interfacial forces. We used the following abbreviations to denote the different interfaces: c-r, the interface between the carrier and the reagent; c-t, the interface between the carrier and the third liquid; t-r, the interface between the third liquid and the reagent.

In the system of three liquids (carrier, reagent, and the third liquid), engulfing and non-engulfing correspond to the presence of different liquid–liquid interfaces. To prevent engulfing (Figure 2b), both the c-r and c-t interfaces must be present. The t-r interface may or may not be present. In the case of engulfing, either of the two interfaces is missing: the c-r interface (third liquid engulfs the reagent plug, Figure 2d,e) or the c-t interface (reagent engulfs the third liquid plug).

By comparing the interfacial tensions (γ), we predicted that engulfing will occur if either of these two inequalities is satisfied: $\gamma_{c-t} > \gamma_{t-r} + \gamma_{c-r}$ or $\gamma_{c-r} > \gamma_{t-r} + \gamma_{c-t}$. Figure 2b shows a three-phase contact line along which the three interfacial forces balance. The three interfacial forces per unit length of the contact line correspond to γ_{c-t}, γ_{c-r}, and γ_{t-r}. This balance requires the vectors corresponding to the three forces to be at equilibrium, which occurs only if the magnitude of every force is smaller than the sum of the magnitudes of the other two forces: $\gamma_{t-r} < \gamma_{c-t} + \gamma_{c-r}$ and $\gamma_{c-r} < \gamma_{c-t} + \gamma_{t-r}$ and $\gamma_{c-t} < \gamma_{t-r} + \gamma_{c-r}$. This case is non-engulfing, because both c-r and c-t interfaces are present. If any of the three inequalities is not satisfied, the interfacial forces cannot be balanced, and one interface would be missing. For example, if $\gamma_{t-r} > \gamma_{c-t} + \gamma_{c-r}$,[37] the net force along the three-phase contact line will cause the s-r interface to shrink and be replaced by a layer of carrier fluid, a situation we define as the s-r interface being wet by the carrier.[38] This case is also non-engulfing, because both the c-r and c-t interfaces are present, and the plug of the third liquid and the reagent plug are completely

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Table 1. Experimentally Tested Predictions of Engulfing Based on Interfacial Tensions[a]

entry	carrier fluid (c)	third liquid (t)	reagent (r)	γ_{c-t}[d] (mN/m)	γ_{c-r}[d] (mN/m)	γ_{t-r}[d] (mN/m)	good spacer? prediction	good spacer? experiment
1	FC3283	SID	water	8.1 ± 0.1	50 ± 2	40 ± 1	N	N
2	FC40	SID	water	8.2 ± 0.1	54 ± 1	40 ± 1	N	N
3	FC3283/ PFO	SID	water	6.3 ± 0.1	16.2 ± 0.3	40 ± 1	Y	Y
4[b]	FC3283	SID	LDAO	8.1 ± 0.1	14.5 ± 0.1	1.5 ± 0.1	N	N
5[b]	FC40	SID	LDAO	8.2 ± 0.1	11 ± 1	1.5 ± 0.1	N	N
6[b]	FC3283/PFO	SID	LDAO	6.3 ± 0.1	10 ± 2	1.5 ± 0.1	N	N
7[c]	FC3283/PFO	SID	LDAO	6.5 ± 0.1	14.4 ± 0.5	8.89 ± 0.03	Y	Y
8	FC3283	DTFS	water	4.2 ± 0.3	50 ± 2	25.8 ± 0.1	N	N
9	FC40	DTFS	water	4.9 ± 0.2	54 ± 1	25.8 ± 0.1	N	N
10	FC3283/PFO	DTFS	water	4.0 ± 0.2	16.2 ± 0.3	25.8 ± 0.1	Y	Y
11	FC40	DTFS	LDAO	4.9 ± 0.2	11 ± 1	16 ± 2	Y	Y
12[c]	FC40	DTFS	LDAO	4.5 ± 0.2	20.0 ± 0.3	14.3 ± 0.1	N	N
13	FC3283/PFO	DTFS	LDAO	4.0 ± 0.2	10 ± 2	16 ± 2	Y	Y

[a] See text for abbreviations. [b] The value of interfacial tension between SID and LDAO was measured over a period of less than 10 min. After the plugs of LDAO and SID were kept in contact for several minutes in the capillary, a change from engulfing to non-engulfing was sometimes observed, presumably due to changes in interfacial tensions as described in the text. [c] The carrier fluid, the reagent, and the third liquid were pre-equilibrated before interfacial tension measurements. [d] The values of interfacial tensions (γ) are presented as an average ± one standard deviation based on four measurements.

separated by the carrier. However, if $\gamma_{c-t} > \gamma_{t-r} + \gamma_{c-r}$, the c-t interface will be wet by the reagent and will not be present; if $\gamma_{c-r} > \gamma_{t-r} + \gamma_{c-t}$, the c-r interface will be wet by the third liquid and will not be present. Both of these two are engulfing conditions. Similar analysis of interfacial tensions in three-phase flow has been previously used to understand the spontaneous motion of liquid slugs in a tube, where the three phases were a gas phase and two liquid phases that both wet the tube.[39,40] While our analysis focuses on balancing interfacial tensions at equilibrium, this analysis[39,40] may be useful to understand nonequilibrium effects that may arise in this system during flow.

To test these two criteria for engulfing, we measured the interfacial tensions for 11 combinations of carrier fluid, reagent, and third liquid, and visualized the reagent−third liquid interfaces in a Teflon capillary. Because we wished to identify third liquids that can be used for protein crystallization in microfluidic plugs, fluorinated oils were chosen as the carrier fluids for their compatibility with protein crystallization.[4,5] Water was used to mimic the reagent, because most protein crystallization is performed in aqueous solutions. We also tested a 0.1% aqueous solution of a detergent, LDAO, as the reagent, because detergents are usually used to solubilize membrane proteins.[41] The use of detergents does not automatically solve the problem of plug coalescence, because the concentration and type of detergents are important parameters for the crystallization of membrane proteins and cannot be adjusted to stop coalescence.

We chose SID and DTFS as candidates for third liquids (Scheme 1). Both liquids are likely to provide high interfacial tensions with water and should be stable under typical conditions for protein crystallization. SID is a disiloxane bearing two phenyl groups. We chose it over other methyldisiloxanes, because it is less likely to swell PDMS microfluidic devices used for protein crystallization. DTFS is a partially fluorinated diester chosen for its likelihood of having a low value of γ_{c-t}. We focused on easily accessible, commercially available liquids. We did not consider hydrocarbon oils due to their tendency to denature proteins and their potential for swelling PDMS. Teflon capillaries were used to ensure that the fluorinated carrier fluid always preferentially wet the channel as a result of the low interfacial tensions between Teflon and fluorinated oils.

In all the cases, the criteria of interfacial tensions correctly predicted whether engulfing happened (bad spacer, N) or did not happen (good spacer, Y) (Table 1). From these measurements, we identified combinations of liquids satisfying non-engulfing conditions. SID plugs were good spacers when FC3283/PFO (10:1, v:v) was used as the carrier fluid. SID plugs were bad spacers for water plugs if the carrier fluid was FC3283 or FC40. Similarly, DTFS plugs were good spacers for both water plugs and plugs of 0.1% LDAO only if the carrier fluid was FC3283/PFO (10:1, v:v).

To be predictive, interfacial tensions must be measured over a period of time to account for potential cross-reactivity of liquids and extraction of components from one liquid to another. When DTFS was used with 0.1% LDAO as the reagent and FC40 as the carrier, DTFS did not engulf a plug of 0.1% LDAO until the two liquids were kept in contact for several minutes. To understand this change from non-engulfing to engulfing, we measured the interfacial tensions before and after the DTFS and 0.1% LDAO were brought into contact. Our results indicated that the DTFS/FC40 and DTFS/LDAO interfacial tensions remained constant, while the interfacial tension between FC40 and LDAO increased from 11 to 20 mN/m in the two experiments (Table 1, entries 11 and 12). Similarly, the interfacial tension between SID and LDAO increased from 1.5 to 8.9 mN/m over long-term contact between the two phases, and we observed a change from engulfing to non-engulfing in the three-phase system of FC3283/PFO, SID, and LDAO (Table 1, entries 6 and 7). These changes in interfacial tension may be attributed to the extraction of LDAO by DTFS and SID, and they could explain the observed changes of engulfing behavior. We have not tested this hypothesis.

To confirm that plugs of the identified non-engulfing third liquids were indeed effective as spacers, we performed experiments using SID plugs to separate aqueous plugs of different viscosities in Teflon tubing (Figure 3a). Without SID plugs, the viscous and nonviscous reagent plugs quickly coalesced after traveling in the channels for less than 10 cm. Upon insertion of SID plugs, the four plugs came together (as shown in Figure 3a) as a result of relative motion but remained in this state without coalescing. Although the plugs in Figure 3a were visualized after traveling ∼20 cm, we did not see any changes in the plugs until they exited the channel (∼40 cm). While flow rates of less

(38) If the wetting condition is barely met and a thin film of micrometer thickness is formed as a result of wetting, purely considering the interfacial tensions may not be enough. In this case, the disjoining pressure described in this review becomes important: Bergeron, V. *J. Phys.: Condens. Matter* **1999**, *11*, R215−R238.

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than 10 μL/min are typically used for protein crystallization experiments, our experiments indicated that SID plugs were effective spacers under flow rates up to 40 μL/min (the highest flow rates tested). In these experiments, the plugs were flowing in Teflon capillaries with circular cross section. In commonly used PDMS channels with rectangular cross sections,[31,32] relative motion, and therefore the coalescence of plugs, may be easier than in circular capillaries, as a result of the thicker layer of carrier fluid in the corners of the channels with rectangular cross sections. To test spacers in square channels,[42] we generated alternating plugs of viscous and nonviscous solutions separated by SID plugs and injected the plugs into a silanized PDMS device. This device was previously designed and used to split an array of large 160-nL plugs into eight arrays of smaller (~20-nL) plugs.[17] The plugs flowed smoothly through the square PDMS channels, and every plug, including the spacers, was evenly split into two at each splitting junction (Figure 3b). The viscous and nonviscous plugs remained separated by SID spacer plugs throughout the process. This experiment demonstrated that (i) SID plugs can also act as spacers in square channels made of PDMS, and (ii) reagent plugs separated by SID plugs can be manipulated and split in PDMS devices.

We also tested whether SID was compatible with injection using a T-junction microfluidic device. A major application for the third liquid could be to separate plugs of different reagents with different viscosities in pre-loaded cartridges.[28] For applications ranging from protein crystallization[6] to chemical screening[15] to enzymatic assays,[6] plugs from the cartridge need to be injected with a stream of a substrate solution using a T-junction (Figure 4a). We formed an array of alternating plugs of viscous (colorless) and nonviscous (red) aqueous solutions separated by a SID plug (Figure 4b). This array of plugs was combined with a stream of colorless substrate solution through a T-junction, as described previously.[43] As shown by the colors of plugs in Figure 4b, the viscous and nonviscous plugs were separated by SID plugs before and after injection. No coalescence or cross-contamination between the plugs occurred in this process, and every aqueous plug in the array was injected with a constant volume of the substrate solution, which was verified by comparing the lengths of plugs before and after injection (Figure 4b). These experiments confirmed that the reagent plugs separated by SID plugs could be manipulated in channels without problems and are compatible with injection in a T-junction.

To ensure that SID is also compatible with crystallization of proteins, we crystallized a human Tdp1 protein in the presence of SID plugs. Tdp1 (tyrosyl-DNA phosphodiesterase 1)[44,45] is an eukaryotic enzyme that hydrolyzes the tyrosine-DNA phosphodiester linkage,[44] and the crystal structure of this protein has been previously reported.[46] We formed alternating plugs of SID and the crystallization solution (22% PEG-3000, 0.2 M NH_4Ac, 0.1 M HEPES buffered at pH 7.5) in a Teflon capillary and injected the plugs with a stream of Tdp1 solution through a T-junction.[6,15] Crystals of Tdp1 appeared in the resulting plugs after incubation for 4 days, indicating that the spacer does not cause obvious problems for protein crystallization, at least for this protein. To use SID extensively for protein crystallization, the interactions between SID and common crystallization reagents should be characterized. Such interactions include the solubility of organic additives in SID, the stability of SID over long-term contact with acidic or basic reagents, the stability of proteins in contact with SID, and the possible loss of proteins into SID. We have performed preliminary experiments indicating that SID was stable when placed next to aqueous plugs of pH typical to protein crystallization (pH ~ 4.5 to 8.5), but a detailed examination of this issue is beyond the scope of this paper. Membrane proteins are solubilized using detergents, and their crystallization in plugs requires special handling.[47] We did not demonstrate the use of third liquids for crystallization of membrane proteins, because the necessary detailed characterization of potential problems such as detergents being extracted by the third liquid is beyond the scope of this paper.

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Conclusions

Here, we showed that plugs of an immiscible third liquid could be used as spacers to prevent adjacent reagent plugs from coalescing in microfluidic flow. We identified and experimentally tested the criteria of interfacial tensions for engulfing or non-engulfing conditions. We found that only plugs of third liquids satisfying the non-engulfing conditions ($\gamma_{c-r} + \gamma_{t-r} > \gamma_{c-t}$ and $\gamma_{c-t} + \gamma_{t-r} > \gamma_{c-r}$) were effective spacers. Two liquids, SID and DTFS, were found to satisfy non-engulfing conditions when used with fluorinated carrier fluids and aqueous reagent plugs. Plugs of SID were tested and found effective as spacers in both circular Teflon capillaries and PDMS channels with square cross sections. SID plugs were compatible with injection using a T-junction, splitting using PDMS devices, and crystallization of a soluble protein.

With the non-engulfing conditions described in this paper, identifying new spacers for protein crystallization and other applications of microfluidic droplets can be facilitated by considering the interfacial tensions between the third liquid, the carrier fluid, and the reagent. The non-engulfing conditions are based on two assumptions that are usually satisfied in the formation of microfluidic plugs: (i) only the carrier fluid wets the channel, and (ii) the capillary number is small.[35] If the reagent also wets the channel, a situation not described in this paper, the interfacial tensions between the channel and the reagent have to be considered in addition to the considerations presented here. At high values of *Ca*, viscous forces need to be considered to account for potential deformation and breakup of plugs. In general, to understand suitability of a third liquid for a particular application, the interactions between the third liquid and the reagents will have to be characterized. We did not perform exhaustive characterization of the compatibility of the two liquids analyzed here, SID and DTFS, with all reagents used in protein crystallization. Identifying and validating ideal spacers for protein crystallization is a pressing issue, especially for crystallization of membrane proteins.[47] As all of these issues are addressed, this work should become useful for a wide range of applications that involve handling large arrays of droplets in microfluidic channels.

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