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PAPER

Microfluidic platform for combinatorial synthesis in picolitre droplets†

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This paper presents a droplet-based microfluidic platform for miniaturized combinatorial synthesis. As a proof of concept, a library of small molecules for early stage drug screening was produced. We present an efficient strategy for producing a 7 × 3 library of potential thrombin inhibitors that can be utilized for other combinatorial synthesis applications. Picolitre droplets containing the first type of reagent (reagents A_1, A_2, …, A_m) were formed individually in identical microfluidic chips and then stored off chip with the aid of stabilizing surfactants. These droplets were then mixed to form a library of droplets containing reagents A_{1-m}, each individually compartmentalized, which was reinjected into a second microfluidic chip and combinatorially fused with picolitre droplets containing the second reagent (reagents B_1, B_2, …, B_n) that were formed on chip. The concept was demonstrated with a three-component Ugi-type reaction involving an amine (reagents A_{1-3}), an aldehyde (reagents B_{1-7}), and an isocyanide (held constant), to synthesize a library of small molecules with potential thrombin inhibitory activity. Our technique produced 10^6 droplets of each reaction at a rate of 2.3 kHz. Each droplet had a reaction volume of 3.1 pL, at least six orders of magnitude lower than conventional techniques. The droplets can then be divided into aliquots for different downstream screening applications. In addition to medicinal chemistry applications, this combinatorial droplet-based approach holds great potential for other applications that involve sampling large areas of chemical parameter space with minimal reagent consumption; such an approach could be beneficial when optimizing reaction conditions or performing combinatorial reactions aimed at producing novel materials.

Introduction

Early stage screening for drug discovery, with its inherently low success rate, demands the capability to efficiently screen large numbers of compounds with both high speed and minimal reagent consumption. Combinatorial chemistry is often used as a method for creating structurally diverse libraries, and robotic systems are used to increase the throughput of both synthesis and subsequent screening.[1,2] Droplet-based microfluidics offers many advantages[3–6] over conventional microtiter plate techniques that could provide powerful enhancements to platforms for combinatorial chemistry for early stage drug discovery. Using droplets of picolitre volume as microreactors allows for individual experiments using six to eight orders of magnitude less starting material than microtiter plate approaches, greatly reducing reagent consumption and improving efficiency through automation and online analysis. In addition to the benefits of miniaturization and increased throughput afforded by droplet-based microfluidics, a large repertoire of downstream microfluidic droplet manipulation and detection techniques allows complex multi-stage experiments with multi-dimensional analysis.[3–6] Interesting biological applications of droplet-based microfluidics have been demonstrated, including single-cell analysis[7–12] and directed evolution of enzymes.[13] The ability to manipulate droplets flowing in immiscible fluids within microfluidic channels also shows potential in chemical synthesis, where the technique has been used for precipitate forming reactions,[14] phase-transfer reactions,[15,16] and biphasic catalysis.[17] Most of these applications in chemical synthesis use identical droplets and do not exploit the opportunity to use droplets to perform multiple different synthetic reactions in parallel.

The high throughput and possibilities for process automation enabled by droplet-based microfluidics hold great promise for its application to combinatorial chemistry. Elegant methods for droplet and plug loading using automation have been demonstrated.[8,18–25] However, current methods lack the capability to

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† Electronic supplementary information (ESI) available: Thrombin enzyme kinetics measured in droplets, additional LC-MS characterization of bulk and droplet-based Ugi-type reaction products, and high speed videos showing droplets immediately after production and reinjected after storage off chip. See DOI: 10.1039/c2lc21019c

efficiently merge two libraries of reagents to create a combinatorial product library. Further developments are needed to extend these capabilities to loading two different libraries of reagents (A_1, A_2, …, A_m and B_1, B_2, …, B_n) and subsequently producing all of the possible $A_{1-m} \times B_{1-n}$ combinations. Combining reagents from libraries A_{1-m} and B_{1-n} to provide all reaction combinations cannot be achieved efficiently using standard methods where droplets are kept single file in microfluidic channels, which have typically been previously used for synthesis in droplets.

Herein, we demonstrate a droplet-based microfluidic platform for combinatorial library synthesis of potential drug candidates. We present an efficient strategy for producing a 7 × 3 library of potential enzyme inhibitors. The concept was demonstrated using an Ugi-type three-component reaction involving an amine (reagents A_{1-3}), an aldehyde (reagents B_{1-7}), and an isocyanide (kept constant). Previous studies have discovered potent thrombin inhibitors by screening crude Ugi reaction products using conventional microtiter plate technology; 'hits' from initial screens were subsequently resynthesized and purified for further analysis.[26,27] Our goal was to increase the efficiency with which initial screens on crude reaction products can be conducted and enable reactions to be carried out with reduced quantities of reagents. Using droplet-based microfluidics, Ugi reactions were compartmentalized into picolitre volumes, using only 10^{-13} mol of reagents per droplet, six orders of magnitude lower than previous microtiter plate synthesis. Droplets containing the first type of reagent (reagents A_1, A_2, …, A_m) were formed individually in poly(dimethylsiloxane) (PDMS) microfluidic devices and then mixed to form a library of droplets containing reagents A_{1-m}. This library was then combinatorially fused with droplets containing the second reagent (reagents B_1, B_2, …, B_n). The library, compartmentalized in droplets, is then ready for further downstream applications such as enzymatic assays to screen for biological activity. This platform could also be used for applications outside of medicinal chemistry, for example combinatorial synthesis aimed at producing novel materials. Our approach could also provide an efficient way to screen different reaction conditions, allowing for many parameters to be manipulated simultaneously to determine the ideal combination of reagents. In summary this technique allows large areas of chemical parameter space to be sampled with minimal reagent consumption.

Materials and methods

Reagent solutions

All reagents were used as received. Starting materials for the Ugi-type reaction came from the following suppliers: **1**, **2**, **4**, **5**, **9**, and **10** from Aldrich; **6**, **7**, and **8** from Acros Organics; **3** from Alfa Aesar; and isocyanide **11** from Specs. Amines **8** and **10** were sold as a dihydrochloride, and **9** was sold as a sulfate salt. Solutions for amine droplet formation contained 100 mM of amine (**8–10**) and 100 mM triethylamine (TEA) in 70% methanol and 30% water, except for **9** where 60% methanol and 40% water were used due to solubility. Aldehyde/isocyanide solutions contained 50 mM aldehyde (**1–7**) and 50 mM isocyanide (**11**) in 60% methanol, 30% water, and 10% triethylene glycol monomethyl ether.

Plug loading

Plugs of the aldehyde/isocyanide solution (15 μL) were loaded into PTFE tubing (0.30 mm ID, 0.76 mm OD, Fisher) connected to a 0.4 × 16 mm Neolus needle (Terumo) and a glass syringe (Hamilton) using a syringe pump. Aqueous reagent plugs were separated by 5 μL plugs of 0.05 wt% EA-surfactant (RainDance Technologies), which is a polyethylene glycol–perfluoropolyether (PFPE–PEG–PFPE) amphiphilic tri-block copolymer,[28] in Fluorinert FC-40 (3M).

Device fabrication

Microfluidic channels were fabricated in PDMS (Sylgard 184, Dow Corning) using soft lithography.[29] PDMS features were bound to a glass slide (Millipore) by exposing the surfaces to oxygen plasma and bringing them into contact. Glass slides coated with indium tin oxide (ITO) on the unbound side (Delta Technologies) were used in all experiments involving electrocoalescence. After heating in a 65 °C oven for 15 minutes, the channels were treated with 2 wt% 1*H*,1*H*,2*H*,2*H*-perfluorodecyltrichlorosilane (ABCR) in HFE-7100 (3M), flushed with Fluorinert FC-40, and placed in a 65 °C oven for at least 1 h. Channels were further treated with 2 wt% 1*H*,1*H*,2*H*,2*H*-perfluorooctyldimethylchlorosilane (ABCR) in HFE-7100, flushed with FC-40, and placed in a 65 °C oven for at least 30 minutes. Electrodes were fabricated with low-temperature solder (Indalloy no. 19, 51% In/32.5% Bi/16.5% Sn, Indium Corporation) using microsolidics.[30,31]

Device operation

The device was imaged using a Phantom v4.2 high-speed digital camera (Vision Research) mounted on an Axiovert 200 inverted microscope (Carl Zeiss SAS). Fluids were dispensed using glass syringes (Hamilton) with 0.4 × 16 mm Neolus needles (Terumo) connected to PTFE tubing (0.30 mm ID, 0.76 mm OD, Fisher). The tubing was inserted into inlet holes in the PDMS device. Flow rates were maintained using Harvard Apparatus PHD 22/2000 syringe pumps.

For amine droplet formation flow rates were adjusted in the following ranges to form droplets with a volume of 0.8 pL: 10–12 μL h^{-1} (aq) and 120–140 μL h^{-1} (oil). The following continuous (oil) phase was used for droplet formation: FC-40 containing 2 wt% EA-surfactant and 1 wt% of a novel fluorinated surfactant, comprised of a dimorpholinophosphate (DMP) polar head and PFPE tail (synthetic details to be described elsewhere). Amine droplets were collected and stored at 4 °C in RDT 1000 Input/Output Vials (RainDance Technologies) containing 3 wt% EA-surfactant in FC-40 (Fig. 1a). The Input/Output Vials can be interfaced with microfluidic devices, syringes, and fluidic connectors, allowing collection and reinjection of droplets. Amine droplets were mixed through a 250 μm bore PEEK cross (VICI), into a collection vial, at 50 μL h^{-1} (Fig. 1b). Aldehyde/isocyanide droplets were produced on the fusion device (Fig. 2) using 25 μL h^{-1} (aq) and 125 μL h^{-1} (oil) while amine droplets were simultaneously reinjected at 7–12 μL h^{-1} with an oil flow rate of 90–110 μL h^{-1} (flow rates were adjusted in real time to achieve optimal pairing). To aid synchronization of droplet pairs, an excess (~20 to 25%)

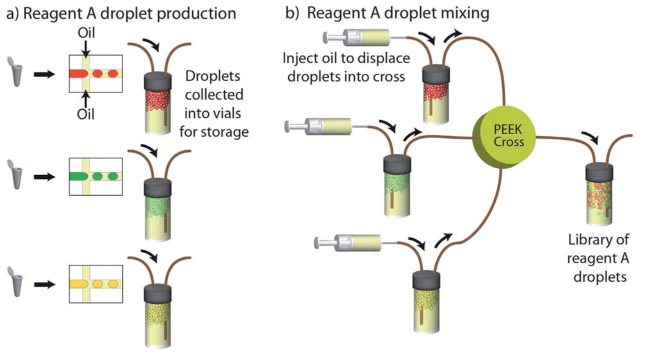


Fig. 1 Schematic diagram of the creation of a library of droplets containing reagents A_{1-3}. (a) Droplets containing reagents A_{1-3} were formed individually using flow focusing and stored prior to use. (b) The droplets were then mixed using a polyether ether ketone (PEEK) cross to form a library of droplets containing reagents A_{1-3}.

of the larger droplets (containing aldehyde/isocyanide) was maintained, which passed through the electrodes unfused. For electrocoalescence, the voltage was applied using an Agilent A33220A function generator connected to a TREK Mode 623B amplifier (35 kHz, 200–300 V, sine wave).

Droplet collection and analysis of Ugi-type reaction

As shown in Fig. 2, the fusion device had a collection outlet and a waste outlet. After fusion, droplets were collected into PTFE tubing (ID 0.56 mm, OD 1.07 mm, Fisher) at the first device outlet (collection outlet) by active extraction: a 1 mL syringe (Omnifix-F, Braun syringe with 0.6 × 25 mm Neolus needles, Terumo) was connected to the PTFE tubing used for droplet storage, and the plunger was actuated with a syringe pump (in refill mode) to extract droplets into the tubing. The refill rate was 240 μL h^{-1}. When the spacer oil was flowing between each aldehyde/isocyanide plug, the syringe plunger was not actuated, and the back pressure at the collection outlet was high, causing the amine droplets to flow passively to the waste outlet. Additionally, at the end of each aqueous aldehyde/isocyanide plug some of the plug remained near the pillar structures that were used as filters (to prevent particles from clogging the microchannels) at the aqueous inlet and were not completely removed as the spacer oil flowed through the inlet. When the next aqueous plug was introduced to the chip, it merged with the residual aqueous solution. To minimize cross-contamination, we discarded droplets produced from the first 25% of each plug, leading to negligible cross-contamination between plugs. In the future, modified inlet geometries[25] could be used to avoid

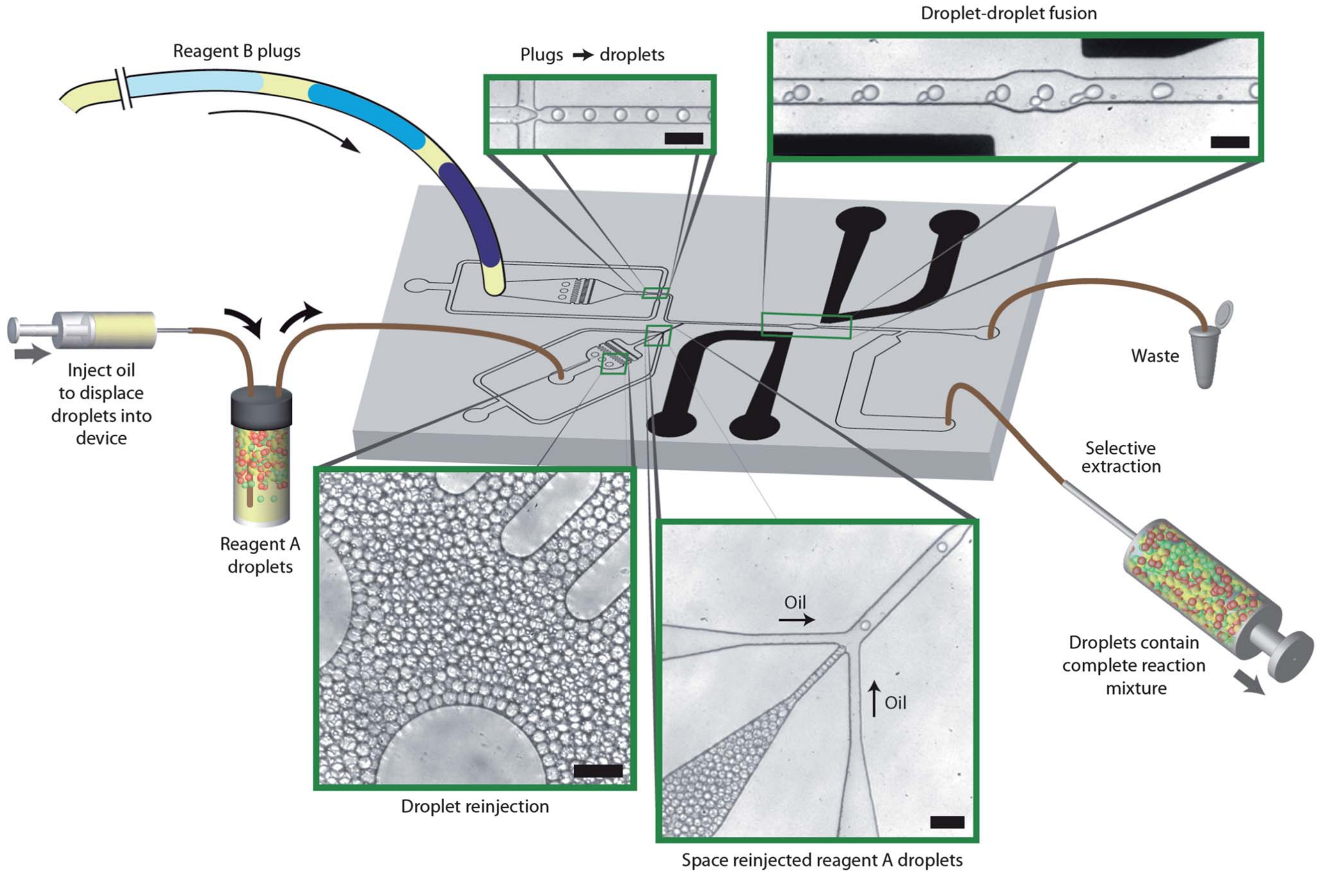


Fig. 2 Schematic diagram of combinatorial synthesis in microdroplets with optical micrographs for key droplet manipulations. Droplets containing reagents B_{1-n} were formed in a PDMS device *via* flow focusing from plugs stored in PTFE tubing. These reagent B_{1-n} droplets were paired with reinjected droplets containing reagents A_{1-m} and fused to produce a library of droplets containing combinatorial mixtures of reagents A_{1-m} and B_{1-n}. Fusion was achieved using electrocoalescence by applying 200–300 V (35 kHz, sine wave). Fused droplets containing the reaction mixture were extracted and stored off chip at room temperature for the duration of the reaction. The scale bars are 50 μm.

residual droplets altogether, thus allowing use of the entire reagent plug.

The collected emulsion was stored in PTFE tubing at room temperature for 24–27 h for the Ugi-type reaction. The emulsions were then broken by adding 30 μL of 1*H*,1*H*,2*H*,2*H*-perfluorooctanol (Aldrich) and 60 μL of methanol to each sample. After centrifuging for 5 min at 13 400 rpm (using an Eppendorf MiniSpin F45-12-11 centrifuge), the aqueous/organic phase was analyzed by LC-MS (Thermo Scientific Accela HPLC with a Surveyor MSQ Plus, Thermo Scientific LCQ Fleet three-dimensional ion trap mass spectrometer). The Ugi-type reactions were repeated in bulk and analyzed by LC-MS. Additionally, bulk reactions were analyzed by HRMS (MicroTOF, Brüker Daltonics) within 20–48 h of reaction. Data are provided in Table S1†.

Results and discussion

Microfluidic platform design and development

To achieve library synthesis in droplets, we integrated multiple droplet manipulation modules. First reagent A_{1-m} droplets (~0.8 pL) were generated separately using flow focusing.[32] 6.3 × 10^7 droplets were produced containing each reagent (at 4 kHz) and stored individually in collection vials containing the continuous oil phase at 4 °C prior to use. Droplets can be used after formation or stored for up to one week. The polydispersity of the droplets at formation was <3%, as is typical for emulsions made using flow focusing,[33] and did not change appreciably over one week. An example of droplets directly after production and reinjected after 5 days of storage is included in the ESI†, indicating good stability after off-chip storage (videos S1 and S2†). Before conducting the combinatorial reaction, droplets containing reagents A_{1-m} were mixed to create a library of droplets (Fig. 1).

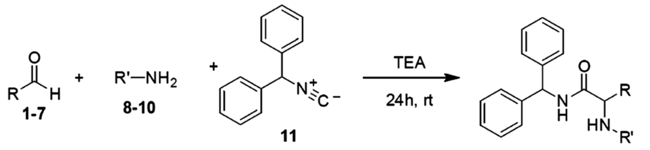
To enable sequential delivery of reagents B_{1-n} to the microfluidic device used for combinatorial synthesis (Fig. 2), reagents B_{1-n} were loaded into plugs within polytetrafluoroethylene (PTFE) tubing. Plugs were formed by sequentially aspirating reagents B_{1-n} (15 μL) and an immiscible fluorinated oil (5 μL) to be used as a spacer phase between each plug. The PTFE tubing containing the plugs was connected to the aqueous inlet of a flow-focusing module (Fig. 2), and ~2.3 pL droplets were formed from the reagent B_{1-n} plugs. Simultaneously, the emulsion containing reagents A_{1-m} compartmentalized into droplets was reinjected into the same device (Fig. 2). Pairs of reagent A_{1-m} droplets and reagent B_{1-n} droplets were then fused using electrocoalescence[34–37] (at 2.3 kHz) producing ~3.1 pL droplets containing all combinatorial mixtures of reagents A_{1-m} and B_{1-n}. Based on the analysis of >500 droplets (using images from 4 different high-speed movies taken at 4 different plugs), 96 ± 3% of droplet pairs fused upon passing through the electric field; of the fused droplet pairs 99 ± 2% represented fusion of a reagent A droplet and a reagent B droplet as intended (1 ± 2% were unintended fusions of two reagent A droplets with one reagent B droplet). Unfused droplets (primarily reagent B droplets which were deliberately maintained in excess; see Materials and methods) made up 37 ± 3% of the final library. The device contained two outlets enabling extraction of fused droplets into PTFE tubing connected to a syringe, which was actuated with a syringe pump in withdraw mode. When the spacer oil was flowing between each reagent B plug, the syringe pump was not actuated and the unfused reinjected reagent A_{1-m} droplets flowed to the waste outlet (see Materials and methods).

This approach minimizes device complexity by taking advantage of off-chip droplet manipulation. If desired, reagent A_{1-m} droplets could all be formed on a single device containing multiple droplet generators, the output of which could be coupled to an electrocoalescence module for fusion with reagents B_{1-n}. The approach presented herein allows for more straightforward device operation by minimizing the number of liquids that must be pumped into a single chip simultaneously.

Ugi reactions executed with droplet-based combinatorial synthesis

To demonstrate the utility of the microdroplet platform for combinatorial drug synthesis, we used the Ugi multicomponent reaction (MCR). MCRs involve the combination of three or more starting materials to form a product.[38] Allowing for the incorporation of wide structural diversity in a single reaction, MCRs are ideal for creating libraries of drug candidates for determining structure–activity relationships in early stage screening and have been used in the synthesis of a variety of drug candidates.[38,39] Here we use a three-component Ugi-type reaction (Scheme 1),[26,27] involving an amine, an aldehyde, and an isocyanide, to synthesize a library of small molecules with potential thrombin inhibitory activity. Thrombin, a serine protease important in blood clotting, is a target for therapeutic anticoagulants.[40] The ability to detect thrombin inhibition from crude Ugi-type reaction mixtures, even in the presence of unreacted starting material, has been demonstrated in previous work conducted in microtiter plates.[26,27]

We performed reactions with aldehydes **1–7** and amines **8–10**, keeping isocyanide **11** constant, to form a 7 × 3 library of possible thrombin inhibitors (Fig. 3). To achieve this synthesis in microdroplets, droplets containing amines **8–10** and equimolar triethylamine (reagents A_{1-3}) were preformed, mixed, and reinjected into a fusion device. Droplets containing aldehydes **1–7** and isocyanide **11** in a 1 : 1 molar ratio (reagents B_{1-7}) were formed from preloaded plugs and fused with the reinjected amine droplets. This 7 × 3 library is a subset of a larger library of thrombin inhibitor candidates previously synthesized and screened for thrombin inhibition by Illgen *et al.* using conventional microtiter plate technology.[27] Although these reactions are typically low yielding (an isolated yield of 18% was achieved in optimized larger scale synthesis of the most potent inhibitor as a racemic mixture[27]), for the purposes of early stage drug screening Illgen *et al.* demonstrated that yield is not essential, discovering novel thrombin inhibitors by first screening crude



Scheme 1 Ugi-type three-component reaction for library synthesis.

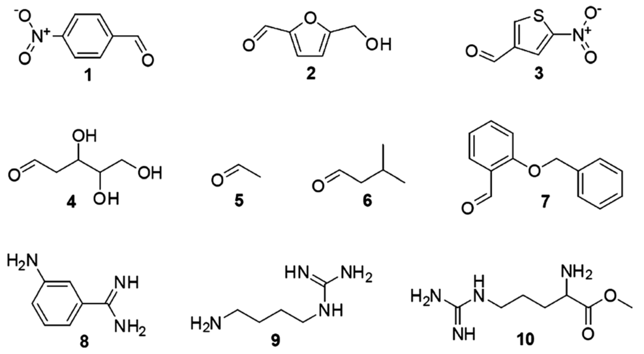


Fig. 3 Aldehyde and amine starting materials for a 7 × 3 combinatorial library.

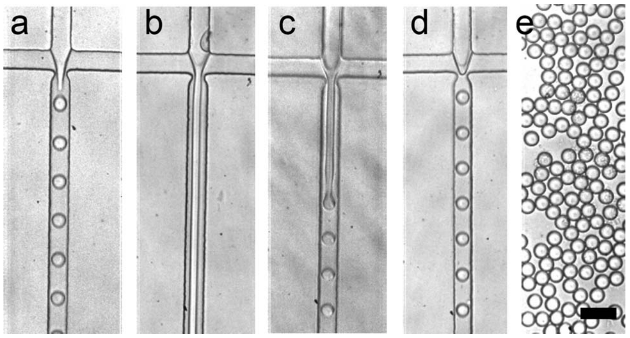


Fig. 4 Development of conditions for solvent droplet formation and stability. The fluorinated continuous phase composition was varied to allow for reproducible formation of droplets stable to coalescence. Droplets contain methanol (60%), water (30%), and triethylene glycol monomethyl ether (10%). The continuous phase was comprised of FC-40 and 3 wt% EA-surfactant (a and b), 3 wt% DMP–PFPE surfactant (c), and 1 wt% DMP–PFPE and 2 wt% EA-surfactants (d and e). Droplets formed with the optimized surfactant mixture are stable to coalescence at the device outlet (e). In (b), Zonyl FSN-100 (1 wt%) was added to the aqueous phase. The flow-rates were 30 μL h^{-1} (aq) and 125 μL h^{-1} (oil) in all cases. The channel surface was treated with trichlorofluorinated silane and monochlorofluorinated silane in all cases. The scale bar is 40 μm. The channels were 15 μm deep.

Ugi-type reaction products for inhibitory activity. Selected 'hits' from the initial screen of crude products were then resynthesized and purified by conventional bulk methods to precisely evaluate potency.[27] The goal of our work is to produce the crude reaction products for the initial screen with greater efficiency and reduced reagent consumption. We chose starting materials that provide a wide range of structural diversity and include a known 2 nM thrombin inhibitor,[27] produced by reacting **7**, **8**, and **11**.

Surfactant formulations for formation of stable solvent droplets

In order to conduct the Ugi-type reaction in droplets, we first developed conditions for the reproducible formation of stable droplets containing the reagents and solvents. We desired to manipulate the droplets both on and off chip, requiring excellent stability to coalescence. We chose to use a fully fluorinated oil, Fluorinert FC-40 (3M), as carrier since perfluorinated oils are immiscible with both aqueous and organic phases, and non-fluorinated compounds are highly insoluble in fluorinated oils,[41,42] preventing partitioning of the compounds into the oil. Fluorinated oil and surfactants have previously been used to produce stable fully aqueous droplets that can be taken off chip and subsequently reinjected.[7,28,43] However, to solubilize the structurally diverse aldehyde starting materials, a solvent mixture consisting of methanol, water, and triethylene glycol monomethyl ether (in a 6 : 3 : 1 volumetric ratio) was used. We therefore developed continuous phase formulations to allow reliable formation of droplets containing this solvent mixture and high droplet stability.

After testing a variety of surfactant formulations (Fig. 4), we found that a mixture of 2 wt% EA-surfactant (RainDance Technologies), which is a polyethylene glycol–perfluoropolyether amphiphilic tri-block copolymer (PFPE–PEG–PFPE),[28] and 1 wt% of a novel fluorinated surfactant, comprised of a dimorpholinophosphate (DMP) polar head and PFPE tail, in FC-40 fluorinated oil allowed for reproducible droplet formation (Fig. 4d) and stability at the device outlet (Fig. 4e). Although droplets could be formed with the surfactant formulations shown in Fig. 4a and c, mild or severe jetting was observed, respectively; thus the point of droplet breakup within the channel was not reproducible. Co-flow resulted when 1 wt% Zonyl FSN-100 surfactant was added to the aqueous phase in addition to 3 wt% EA-surfactant in the continuous phase (Fig. 4b). Together these results highlight the importance of surfactants and co-surfactants in droplet formation as well as stability. Additionally, we found that silanizing the PDMS channels with a trichlorofluorinated silane followed by treatment with a monochlorofluorinated silane (as described in Materials and methods) was necessary for reproducible droplet formation.

Analysis of reaction products

To simplify analysis of the Ugi-type reaction, separate tubing was used to collect droplets formed from each aldehyde plug, resulting in a total of seven samples, each containing droplets with three different reactions. Droplets containing the complete Ugi-type reaction mixture were left to react at room temperature for 24 h, following conditions for the bulk library synthesis.[27] Each of the 21 reactions was also performed under the same conditions in bulk and analyzed with both liquid-chromatography-mass spectrometry (LC-MS) and high resolution mass spectrometry (HRMS) where possible (see ESI, Table S1†).

To evaluate the presence of the desired product, the emulsion was broken by addition of 1*H*,1*H*,2*H*,2*H*-perfluorooctanol (PFO) and methanol, and centrifugation, after the 24 h reaction period. After removal of the fluorinated phase, the reaction mixture was analyzed by LC-MS. LC-MS indicated the presence of the desired product in 20 out of the 21 reactions as shown in Table 1. The one reaction that did not occur in droplets (reaction of **2**, **9**, and **11**) also did not occur when the reaction was performed under bulk conditions. The retention times and masses from the bulk reactions showed close correspondence with those in droplets (typically within 0.05 min and 0.1 Da, respectively), and HRMS showed close correspondence with the predicted values (within the expected error for the analysis method used, see ESI†).

Hence, we have successfully demonstrated a complete droplet-based workflow for loading a library of reagents into droplets,

Table 1 Mass spectrometric analysis of Ugi-type three-component reaction in droplets[a]

	Amine		
Aldehyde	**8**	**9**	**10**
1	480.28 (480.20)	475.31 (475.25)	533.31 (533.25)
2	455.18 (455.21)	—[b]	508.33 (508.26)
3	486.26 (486.16)	481.26 (481.20)	539.30 (539.21)
4	463.33 (463.23)	458.42 (458.28)	516.36 (516.28)
5	373.26 (373.20)	368.27 (368.24)	426.33 (426.25)
6	415.29 (415.25)	410.33 (410.29)	468.34 (468.30)
7	541.31 (541.26)	536.37 (536.30)	594.37 (594.31)

[a] Experimental *m*/*z* values are listed followed by theoretical value in parentheses for the product of the Ugi-type 3-component reaction with the aldehyde and amine indicated and isocyanide **11**. Masses indicated are for the protonated product, detected in ESI positive ion mode. [b] The presence of the desired product was not observed; this is consistent with bulk reaction results.

combining them in a combinatorial manner, and conducting an Ugi-type three-component reaction in droplets.

Conclusions

In summary we have developed a droplet-based microfluidic platform for combinatorial synthesis in picolitre droplets. By combining droplet formation, mixing, reinjection, fusion, and storage modules, we demonstrated combinatorial synthesis, performing a total of 20 Ugi-type reactions in droplets. Droplets of one library of reagents (A_{1-m}) are first taken off chip and mixed such that the droplet mixture can be reintroduced onto a second chip and merged with droplets containing reagents B_{1-n}, created directly on-chip. Sufficient replicate droplets of each reagent were produced to ensure that all reaction combinations arise statistically by droplet fusion. Droplets containing the combinatorial reactions were produced at over 2 kHz. Furthermore, this method drastically reduces reagent consumption, allowing reactions to be conducted in volumes six orders of magnitude lower than in microtiter plates and enabling experiments with precious or costly reagents. The approach is applicable to other combinatorial reactions, allowing high throughput production of diverse chemical structures.

This platform has a number of attractive features. The system used here for a 7×3 combinatorial library is scalable to make larger libraries. Additional reactions can be conducted by producing droplets of additional amines and aldehydes since subsequent fusion and collection of droplets containing the full reaction mixture can be conducted with high efficiency once the reagents are loaded. Automated robotic systems could be used to produce reagent A_{1-m} droplets[44] and load reagents B_{1-n} into nanolitre or microlitre reagent plugs separated by oil into PTFE tubing.[18–20,23–25] To mix larger libraries of A_{1-m} droplets (higher m value), multiple PEEK crosses or mechanical mixing[44] could be used. Furthermore, many droplets containing each reaction are produced; for the 21 member library here 10^6 droplets were produced for each reaction (at a rate of 2.3 kHz). Hence, the droplet library can be divided into aliquots and each aliquot can potentially be used to screen the library under different conditions, for example for inhibitory activity against a variety of enzyme targets.

Screening for enzyme inhibition (or other biological activities) will likely involve using the droplets created with this combinatorial synthesis system to deliver compounds to droplets containing target enzymes (or other proteins or cells) and other assay reagents *via* droplet fusion[34–37] in additional microfluidic systems. Several droplet-based microfluidic systems for measuring enzyme kinetics and enzyme inhibition have already been demonstrated,[3–6,45,46] and we have measured thrombin kinetics in droplets using microfluidic delay lines and high throughput fluorescence measurements to analyze individual droplets (see ESI, Fig. S1†). The throughput of many systems for drop fusion[34–37] and for measuring enzyme kinetics and enzyme inhibition[45,46] is ≥kHz (≥1000 per second), similar to the frequency with which droplets containing each reaction are produced using the system described here (2.3 kHz). For many early screening applications, knowing the identity of the hit may not be as important as determining whether or not hits exist within a given library; in this case, the droplet-based synthesis could be used as a rough initial screen to be followed up by conventional methods if 'hit(s)' are found within a given library. If determining compound identity is important, the compound library could be encoded optically or with particles[8,47] or hits could be sorted[48–50] and studied using advances in single droplet analysis with mass spectrometry.[51–54] Having demonstrated that thrombin assays can be performed in droplets on chip (ESI, Fig. S1†) and that our droplet-based combinatorial synthesis platform successfully generates known thrombin inhibitors *via* an Ugi-type reaction (Table 1), we expect downstream screening for thrombin inhibition in droplets to proceed as observed previously in microtiter plates.[27] The work presented herein describes a complete platform for droplet-based combinatorial synthesis which can be readily integrated with myriad existing analysis and processing techniques downstream.

Acknowledgements

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