Microfluidic Synthesis

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Synchronized Synthesis of Peptide-Based Macrocycles by Digital Microfluidics**

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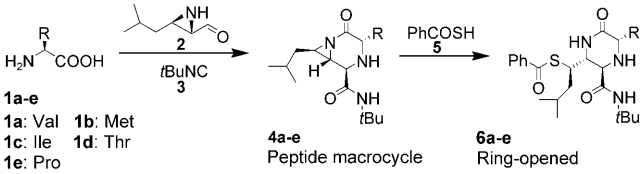
There has been considerable interest in peptide-based macrocycles,[1–5] as their topology allows them to resist digestion by exopeptidases while retaining high affinities for their biochemical targets.[4,6,7] We recently described a novel macrocyclization strategy that is enabled by amphoteric aziridine aldehydes (Scheme 1).[8] Peptide macrocycles can now be



Scheme 1. Synthesis of peptide-based macrocycles and their structurally modified derivatives.

made with high chemoselectivity from amino acids or linear peptides, isocyanides, and amphoteric aziridine aldehydes in a one-step process. Importantly, the resulting products possess useful structural features that allow specific modification at defined positions. In our initial work, we formed serial batches of peptides using conventional macroscale synthetic techniques. The utility of this method is limited, however, without a high-throughput approach for generating focused libraries of peptide macrocycles. Such a method would be automated and would enable multistep reactions to be handled in parallel. Herein, we present a new miniaturized technique for synchronized on-chip synthesis of peptide macrocycles and related products.

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The most common format for miniaturized synthesis is enclosed microchannels in planar platforms. Such systems have been used for conventional organic synthesis,[9–13] polymerization reactions,[14,15] formation of biomolecules, such as peptides and DNA,[16–18] and generation of nanoparticles and colloids.[19–21] However, there are some challenges that limit the scope of their use for parallel chemical synthesis. For example, many microchannel platforms are formed from poly(dimethylsiloxane), a material that is susceptible to degradation in common organic solvents.[22,23] Furthermore, control of many reagents simultaneously (a feature required to implement parallel synthetic reactions) in microchannels requires pumps, tubing, valves, and/or three-dimensional channel networks that can be difficult to fabricate and operate.[9,24] This has prompted researchers to develop specialized techniques[25] to overcome this limitation. Another disadvantage associated with the microchannel format is the challenge inherent in the removal of solvents and re-dissolution of solids that are common steps in synthesis. Solid reagents and products can clog microchannels, making targeted reagent delivery difficult to control. Finally, the small volumes of samples in microchannels makes it difficult to recover them in sufficient quantities for off-line analysis techniques, such as NMR spectroscopy.

In need of a platform capable of generating a) peptide macrocycles for downstream transfer onto functionalized surfaces, and b) spatially resolved macrocyclic peptide products in the solid state, we chose an alternative format for automation of synthesis, called digital microfluidics.[26] In digital microfluidics, discrete nL to μL sized droplets of samples and reagents are controlled in parallel by applying a series of electrical potentials to an array of electrodes coated with a hydrophobic insulator (Figure 1). Digital microfluidics has become popular for biological and chemical applications, including cell culture and assays,[27–29] enzyme assays,[30–32] immunoassays,[33,34] protein processing,[35–40] clinical sample processing and analysis,[41] and synthesis of anisotropic

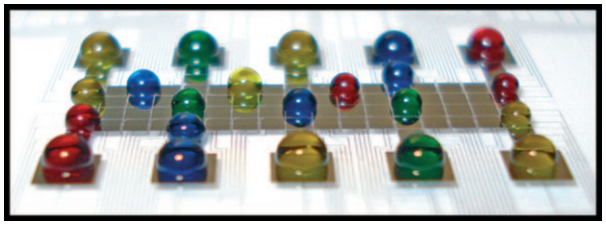

Figure 1. A digital microfluidic platform. Each droplet acts as a microvessel in which parallel reactions can take place with no cross-contamination.

particles and tetrahydroquinolines.[42,43] The initial synthesis studies[42,43] used simple one-plate devices that are capable of carrying out only a single, serial reaction, with no reagent-supply reservoirs, dispensing, splitting, or active mixing. Our goal was to implement a fully integrated platform with reagent supply reservoirs, precise control over reagents (i.e., dispensing, splitting, and active mixing), and the capability to precipitate peptide macrocycles for hierarchical modification and analysis.

Herein, we introduce the first two-plate digital microfluidic platform for chemical synthesis that is suitable for control of many different multi-component, multi-step reactions in parallel. We used this system to carry out synchronized synthesis of peptide macrocycles from three different components. The resulting products contain aziridines as sites that are primed for specific, late-stage modification by nucleophilic ring-opening. Using the same chemistry we demonstrate the synthesis of a nine-membered macrocycle, a ring size that is associated with considerable synthetic difficulties in conventional cyclic peptide synthesis.[44] The new method is fast, amenable to automation, compatible with a wide range of solvents, liberated from external hardware (e.g., pumps, tubing, etc.), and is particularly well-suited for parallel processing.

Parallel synthesis of peptide-based macrocycles was implemented on a new digital microfluidic system (Figure 2a). The design features ten reagent reservoirs and eighty-eight actuation electrodes dedicated to dispensing, merging, and mixing droplets of reagents and products. The devices were designed to handle ten different reagents, including five amino acids, an aziridine aldehyde, *tert*-butyl isocyanide (*t*BuNC), and thiobenzoic acid (PhCOSH) as stock solutions in trifluoroethanol (TFE) or water. As shown in Figure 2b, the digital microfluidic device facilitated the implementation of synchronized synthesis of five different macrocycles in three steps. First, five 900 nL droplets containing one of five amino acid substrates (Val, Met, Ile, Thr, and Pro) were dispensed from their respective reservoirs. Second, five 900 nL droplets containing aziridine aldehyde were dispensed and merged with the amino acid droplets and mixed. Third, five 900 nL droplets of *tert*-butyl isocyanide were dispensed and merged with the droplets containing the amino acids, and incubated. Finally, macrocyclic peptide products were isolated by allowing the solvent to evaporate.

Some macrocycles were further modified in three additional steps to form structurally modified products (Figure 3). First, each macrocyclic peptide product was re-dissolved in fresh trifluoroethanol. Second, five 900 nL droplets containing thiobenzoic acid were dispensed, and merged with macrocycle droplets, and the reactions were allowed to incubate. Third, the aziridine ring-opened products were isolated by allowing the trifluoroethanol to evaporate, delivering precipitated macrocyclic peptide thioesters at defined positions. In all, this method constituted thirty processing steps—six steps each for five reactions in parallel.

Mass spectrometry (MS) and NMR were used to evaluate the effectiveness of on-chip peptide macrocycle and aziridine ring-opened product synthesis. Figure 4 shows representative mass spectra generated from unreacted methionine (Met) and

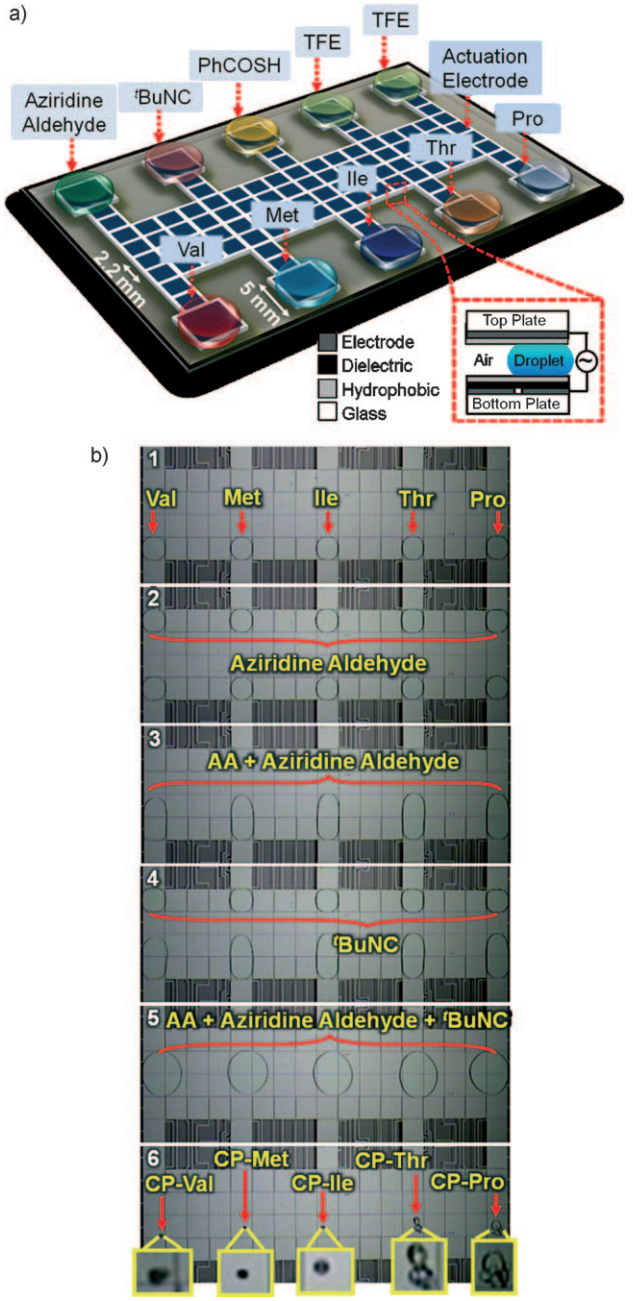

Figure 2. a) Top and side views of the digital microfluidic device used for peptide-based macrocycle (PM) synthesis. b) Sequence of frames from a movie illustrating digital microfluidic-based synthesis of PM. In frames 1–3, droplets (each 900 nL) containing amino acid (AA) substrates and aziridine aldehyde were dispensed from their respective reservoirs, merged, and mixed. In frames 4, 5, droplets of *t*BuNC were dispensed and merged with the droplets of AA substrates and aziridine aldehyde, and the reaction was allowed to proceed for 1 h at room temperature. Finally, in frame 6, PM products were isolated by allowing the solvent to evaporate. The insets in frame 6 are magnified images of the dried products. In these frames, the top plate is not visible, as it is formed from transparent ITO glass.

also peptide-based macrocycle-containing Met and its aziridine ring-opened derivative synthesized on a digital microfluidic device, with peaks at *m*/*z* 150, 342, and 480, respectively (mass spectra of the other peptide-based macrocycles

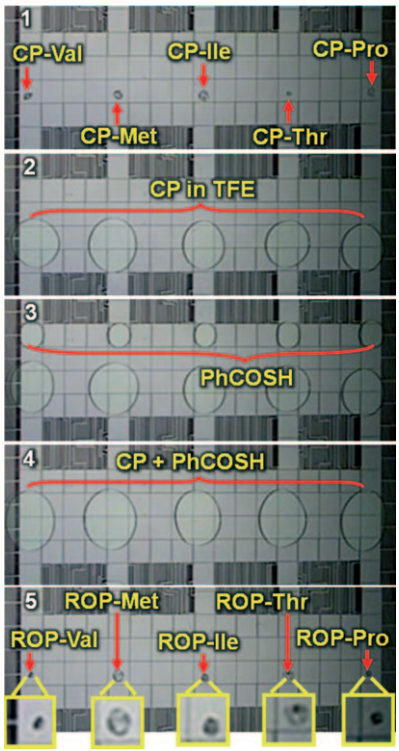

Figure 3. Sequence of frames from a movie illustrating the key steps in digital microfluidic-based synthesis of aziridine ring-opened (RO) products. Peptide-based macrocycles (PM) are solubilized in trifluoroethanol (frames 1, 2), then merged (frames 3, 4) with droplets containing thiobenzoic acid (PhCOSH), and then mixed and incubated for 1 h at room temperature, followed by isolation of RO products (frame 5) by allowing the trifluoroethanol solvent to evaporate. The insets in frame 5 are magnified images of the dried products.

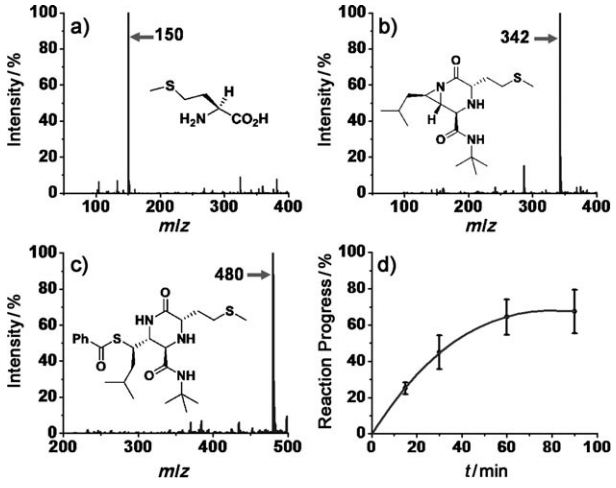

Figure 4. ESI-MS spectra of a) methionine (Met), and products synthesized by digital microfluidics, including a b) peptide-based macrocycle containing Met and c) the aziridine ring-opened derivative. d) Reaction progress by digital microfluidics as percentage conversion of Met over time. Each data point represents the mean ± standard deviation of 4 samples.

and their aziridine ring-opened products are given in the Supporting Information, Figures S1–S4). The mass spectra of products from the microscale synthesis were nearly identical to those of the same products from macroscale synthesis (Supporting Information, Figure S5 a,b). Complementing the MS data, NMR spectra of the peptide-based macrocycle containing methionine (Supporting Information, Figure S6) and its aziridine ring-opened derivative (Supporting Information, Figure S7) synthesized on the digital microfluidic platform and on the macroscale had similar chemical shifts that correlated well with all the protons. The reaction progress was monitored as a function of Met conversion over time on the digital microfluidic platform (Figure 4 d) and on the macroscale (Supporting Information, Figure S5 c). The reaction kinetics of the two methods were similar, with about 70 % (digital microfluidic) and about 90 % (macroscale) depletion of the initial reagent within one hour; we anticipate that future systems integrated with continuous, rapid mixing[45,46] will improve the kinetics of the microfluidic technique.

To further show the application of the technique, a nine-membered macrocycle was synthesized on-chip (Figure 5 a). In this method, a Pro–Leu-derived macrocycle was prepared

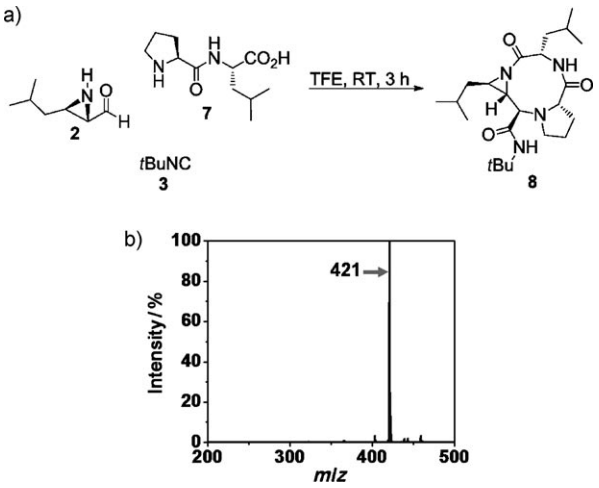

Figure 5. a) Synthesis of a nine-membered macrocycle. TFE = trifluoroethanol. b) ESI-MS spectrum of **8**.

in three steps. 900 nL droplets containing dipeptide, aziridine aldehyde, and isocyanide were dispensed from respective reservoirs, merged, mixed, and incubated. Figure 5 b shows a mass spectrum from a Pro–Leu-derived macrocycle synthesized on a digital microfluidic device, which further demonstrates the reaction selectivity. Of note is an exciting possibility offered by this new on-chip conjugation strategy: thioesters are well-known precursors to native chemical ligation.[47] We anticipate applications that will take advantage of S-to-N acyl transfer from the thioester to a nucleophilic residue within the macrocycle.

The new digital microfluidic method is capable of synthesizing peptide-based macrocycles and aziridine ring-opened derivatives that are analogous to the macroscale method, as shown by MS and NMR results. In comparison with other miniaturized fluidic technologies, digital microfluidics is particularly well-suited for applications in synthesis, as it allows precise control over multiple reagent phases. Importantly, it supports the critical step of solvent removal

and re-dissolution of product for further processing (Figure 3, frames 1,2). This highlights the flexibility of digital microfluidics: there are no limits on the volume of solvent used to re-dissolve a particular solid (for example, in this method, four droplets of trifluoroethanol representing a combined volume of 3.5 μL were dispensed to facilitate dissolution of each solid macrocycle). The salient features of digital microfluidics for synthesis include individual addressing of all reagents with no need for complex networks of microvalves,[9,24] a chemically inert Teflon-based device surface that diversifies the scope of compatible reagents to include organic solvents and corrosive chemicals, and easy access to reasonably large amounts of products for off-chip analysis (such as simply removing the top plate on a device). Last but not least, the technique will likely be well-suited for evaluating macrocyclic libraries if the solvent is removed by evaporation.[48]

In summary, we present a new microfluidic technique for synchronized synthesis that is applied to formation of peptide-based macrocycles and their analogues with side chains appended during aziridine ring-opening. In future work, we anticipate that access to complex cyclic thioesters should facilitate on-chip ligation. The device was designed to handle diverse reagents and thirty reaction steps, and was capable of forming five products in parallel. The multiplexing demonstrated here is likely just the beginning; we propose that future systems might be capable of synthesis of tens or hundreds of products simultaneously, which would streamline the formation of spatially addressable crystalline peptide-based macrocycles. These advantages suggest that there is significant potential for digital microfluidics for fast and automated synthesis of libraries of compounds for applications such as drug discovery and high-throughput screening.

Experimental Section

To synthesize cyclic peptide-based macrocycles, three 900 nL droplets containing a) amino acid (0.1 M in dionized water, 90 nmol), b) aziridine aldehyde (0.05 M in trifluoroethanol (TFE), 45 nmol), and c) *tert*-butyl isocyanide (0.1 M in TFE, 90 nmol) were dispensed from their respective reservoirs and merged. The pooled droplet was mixed (20 s, RT) by periodically actuating in a circular motion on four electrodes and then incubated (1 h, RT) in a Petri dish sealed with parafilm to minimize evaporation. After the reaction, macrocycles were obtained by removing the top plate and allowing the solvent to evaporate (15 min, RT). After synthesizing and isolating macrocycles, some samples were re-dissolved in an appropriate solvent and collected by pipette for off-chip analyses, while others were subsequently processed on-chip to form aziridine ring-opened peptides. In the latter case, peptide-based macrocycle products were resolubilized by dispensing four droplets of TFE and driving them to the dried spot (combined volume 3.5 μL, 90 nmol). A droplet containing thiobenzoic acid (0.1 M in TFE, 90 nmol) was then dispensed and merged with the resolubilized peptide, and the combined droplet was mixed (20 s, room temperature) and then incubated in a sealed Petri dish (1 h, RT). Finally, the aziridine ring-opened peptides were obtained by removing the top plate and allowing the solvent to evaporate (15 min, RT). For analyses of peptide-based macrocycles and aziridine ring-opened peptides off-chip, isolated samples were resolubilized in 100 μL methanol containing 0.1% formic acid for mass spectrometry, or 250 μL CD_3OD for NMR spectroscopy. The synthesis of the nine-membered macrocycles was similar to that of the [4.1.0] macrocycles. The substrate in this reaction was proline–leucine (0.1 M in TFE, 90 nmol), and the reaction required longer incubation (3 h, RT) and frequent mixing (20 s, every 30 min). For reagents and materials, device fabrication and operation, mass spectrometry, NMR spectroscopy, macroscale synthesis, and conversion analysis, see the Supporting Information.

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