Lab-on-a-Chip

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Integrated Microfluidics for Parallel Screening of an In Situ Click Chemistry Library**

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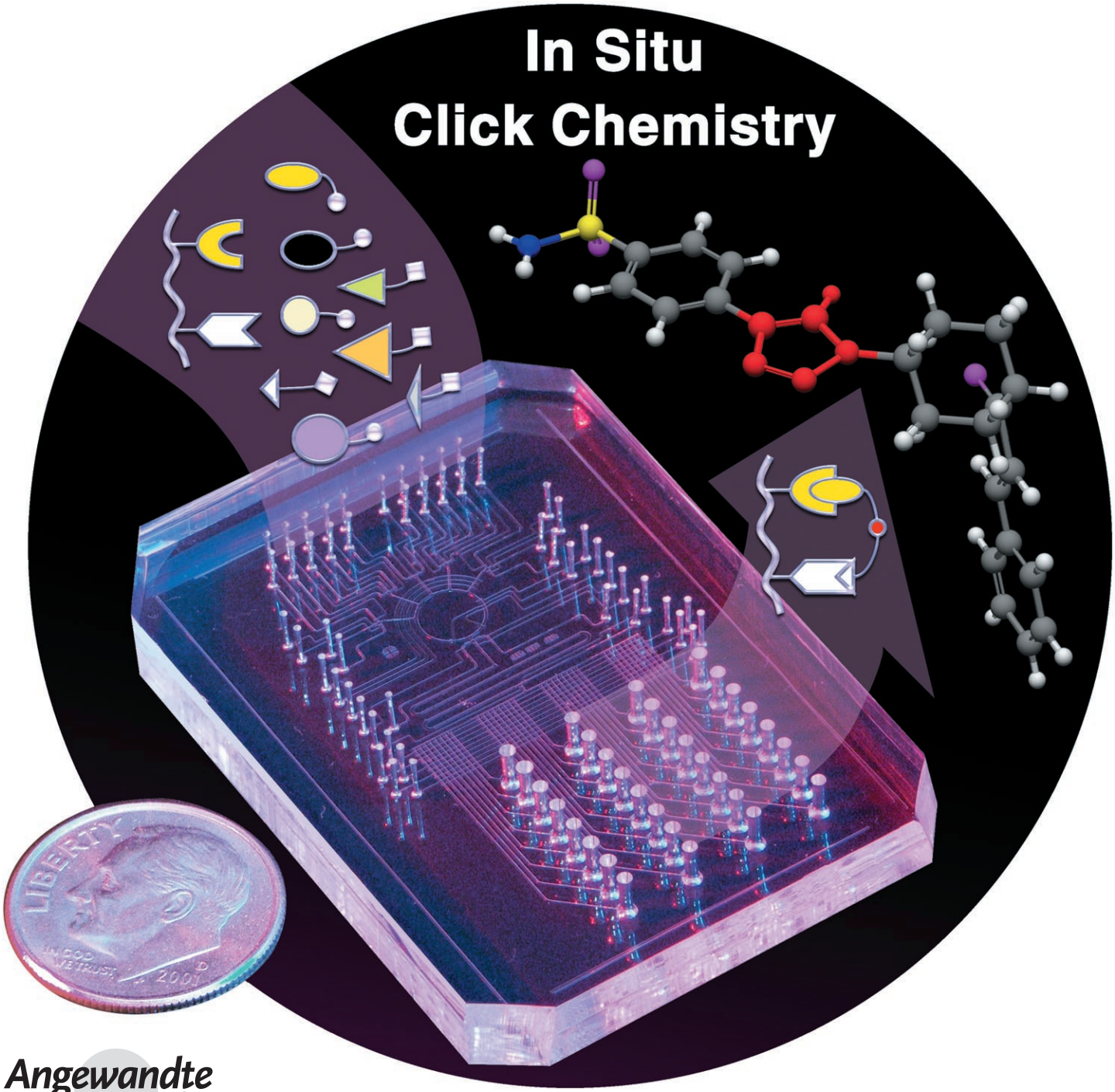
Dedicated to Professor Tien-Yau Luh



There is growing interest in performing chemical reactions in microfluidic devices[1–3] because they offer a variety of advantages over macroscopic reactors, such as reduced consumption of reagents, high surface-area-to-volume ratios, and improved control over mass and heat transfer. Organic reactions[4,5] that involve highly reactive intermediates often exhibit greater selectivities and specificities in microreactors compared to conventional macroscopic synthesis. Many challenges remain, however, in the development of microreactors for 1) multistep syntheses in which the individual steps require a change in solvents, reagents, and conditions, as well as 2) parallel screening in which similar types of reactions are performed using different combinations of reagents. Significant efforts have been devoted to develop functioning modules to improve the performance of microreactors. For example, various valves[6,7] have been demonstrated to isolate distinct regions and prevent cross-contamination from different reactions in a microchip. Elsewhere, different mixing modules[8,9] have been utilized to overcome diffusion-limited mixing in the turbulence-free microfluidic environment. Also, functioning pumps[7,10] that are capable of delivering and metering fluidic components have been successfully integrated with microchannels. With these functioning modules, it becomes feasible to handle complicated chemical and biological processes in microreactors in an automated fashion. In fact, integrated microreactors have been utilized for sequential syntheses of molecular imaging probes,[9] polymerase chain reaction,[11] protein crystallization,[12] and cell culture.[10]

In situ click chemistry is a target-guided synthesis[13–19] method for discovering high-affinity protein ligands by assembling complementary azide and acetylene building blocks inside the binding pockets of the target through 1,3-dipolar cycloaddition.[19–22] The resulting ligands display much higher binding affinities to the target than the individual fragments, and the identification of a hit is as simple as detecting product formation by using analytical instruments, such as LC-MS.[19,21] Recently, this approach was employed to prepare potent inhibitors for acetylcholine esterase (AchE),[19,22,23] bovine carbonic anhydrase II (bCAII),[21] and HIV protease.[24] Typically, in situ click chemistry experiments are conducted in parallel using 96-well microtiter plates. Inevitably, the experimental conditions result in the consumption of significant amounts of the target proteins and reagents, which hampers the broad application of in situ click chemistry screening, especially when target proteins are difficult to obtain. Moreover, the existing approach relies heavily upon manual operation, which limits screening throughput and fidelity. Integrated microfluidics provides an excellent experimental platform for in situ click chemistry screening because it enables parallelization and automation, and, most importantly, the miniaturization allows an economical use of target proteins and click chemistry reagents.

Herein, we describe a new type of microfluidic chemical reaction circuits (Figure 1) that provide an automated platform for the parallel screening of 32 in situ click chemistry reactions, with reduced consumption of target proteins and reagents. We selected the known bCAII click chemistry system[21] for this proof-of-concept study. Acetylenic benzenesulfonamide (**1**; $K_d = 37 \pm 6$ nM) was used as the reactive scaffold (anchor molecule) for screening a library of 20 complementary azides **2**–**21**. In control experiments, we utilized the active-site inhibitor, ethoxazolamide (**22**; $K_d = 0.15 \pm 0.03$ nM), to suppress the in situ click chemistry reactions.

To determine appropriate reaction conditions for this microfluidics-based in situ click chemistry screening, click reactions[21] between acetylene **1** and azide **2** (known to undergo enzyme-induced product formation) were performed under different conditions to ensure minimum use of enzyme and reagents and yet reliable and reproducible LC-MS signals for the identification of hits. The microfluidic screening platform described here utilizes a reaction volume of approximately 4 μL, which corresponds to 19 μg of enzyme, 2.4 nmol of the acetylene, and 3.6 nmol of the azide for each reaction, instead of the 100 μL of reaction mixture (containing 94 μg of enzyme, 6 nmol of acetylene, and 40 nmol of azide) employed in the conventional approach. Overall, 2–12-fold sample economy was achieved.

Our microfluidic chemical reaction circuit comprises four major functional components (Figure 1). A nanoliter-level rotary mixer (component 1; round-shaped loop) with a total volume of about 250 nL is responsible for selective sampling, precise metering,[7] and rotary mixing of nL quantities of reagents—acetylene **1** (80 nL) and azides **2**–**11** (or **12**–**21**; 120 nL) with/without inhibitors **22** (40 nL)—for each screening reaction. A microliter-level chaotic mixer[8] (component 2) combines the reagent solutions from the rotary mixer with μL quantities of bCAII solution in phosphate-buffered saline (PBS, pH 7.4). A homogenous reaction mixture was generated by chaotic mixing inside the 37.8-mm long microchannel (the gray microchannel in the middle section of Figure 1a). The embedded micropatterns (inset in Figure 1a) induce[8] chaotic advection to facilitate mixing within the relatively short microchannel. The micropatterns were 20% longer than theoretically required[25] to ensure

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

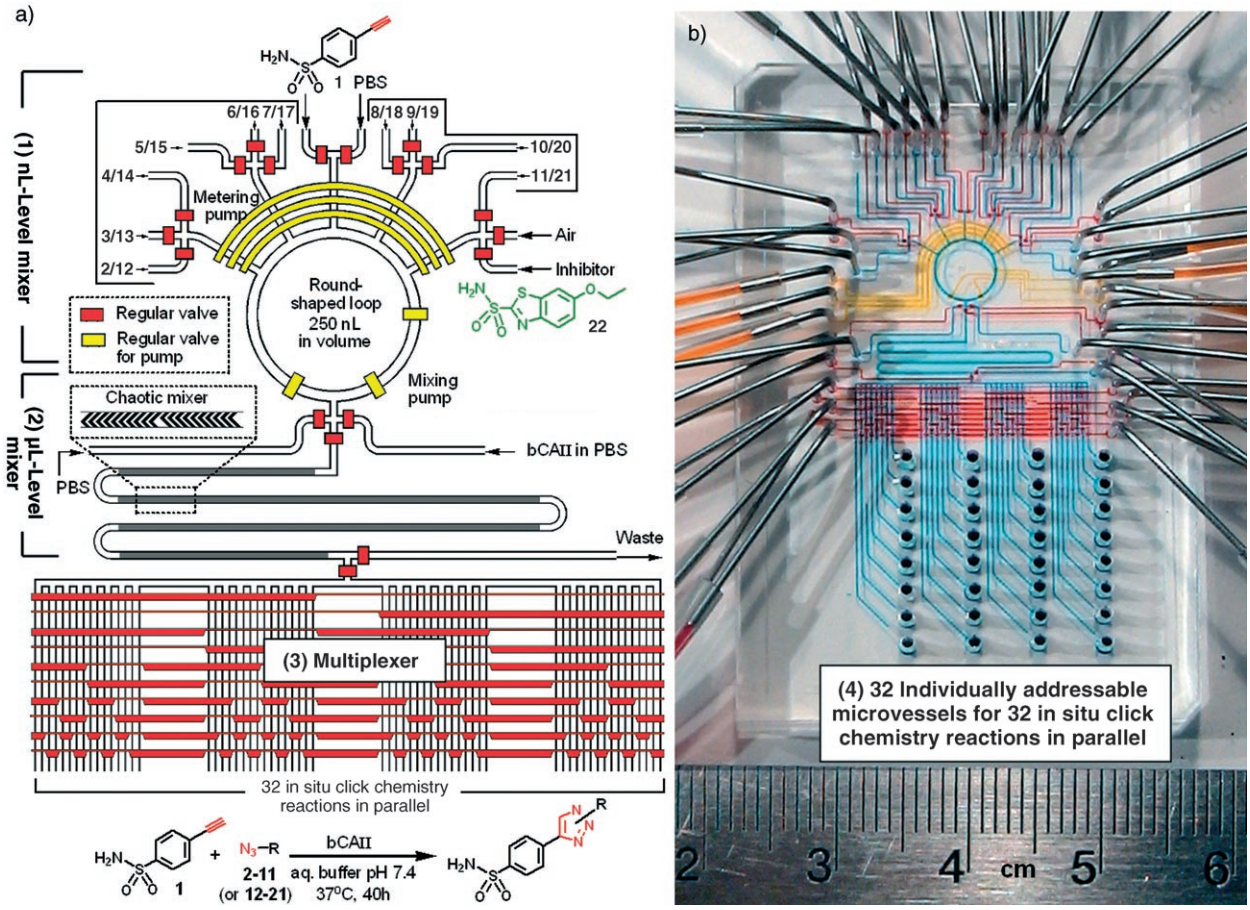


Figure 1. a) Schematic representation of a chemical reaction circuit used for the parallel screening of an in situ click chemistry library. The responsibilities of different valves are illustrated by their colors: red for regular valves (for isolation) and yellow for pump valves (for fluidic metering and circulation). ***n***/***n*** in the nL-level mixer refer to the azides **2**–**11** (or **12**–**21**). b) Optical image of the actual device. The various channels were loaded with food dyes to help visualize the different components of the microfluidic chip: red and yellow as in part (a); blue indicates the fluidic channels. Components (1)–(3) are indicated in part (a), while component (4) is shown in part (b). See text for details.

efficient mixing. Finally, a microfluidic multiplexer[26] (component 3) guides each reaction mixture into one of the 32 individually addressable microvessels (component 4; 1.3 mm in diameter and 6 mm in depth, ca. 8 µL in volume) for storing the reaction mixtures. In situ click chemistry screening of 10 different binary azide/acetylene combinations was performed in parallel by preparing 32 individual reaction mixtures of the following types: 1) ten in situ click chemistry reactions between acetylene **1** and 10 azides in the presence of bCAII; 2) ten control reactions that are performed as in (1), but in the presence of inhibitor **22**, to confirm the active-site specificity of the in situ click chemistry reactions; 3) ten thermal click chemistry reactions performed as in (1), but in the absence of bCAII, to monitor the enzyme-independent reactions; and 4) two "blank" solutions containing only bCAII and PBS solution, respectively. Under these conditions, the entire library of 20 azides **2**–**21** was screened in two batches, first with azides **2**–**11** and then with **12**–**21**. A mixture of DMSO/EtOH (1:4 v/v) was utilized as solvent for all reagents, as it does not damage[27] the PDMS-based microchannels or affect the performance of the embedded valves and pumps. Each in situ click chemistry reaction employed 80 nL of a solution of acetylene **1** (30 mM, 2.4 nmol), 120 nL of a solution of one of the azides **2**–**21** (30 mM, 3.6 nmol), and 3.8 µL of a solution of bCAII (5 mg mL^{-1}, 19 µg) in PBS. For the control reactions, an additional 40 nL of solution of inhibitor **22** (100 mM, 4 nmol) was added. In the thermal reactions, the solutions of bCAII were replaced with PBS.

By means of a computer-controlled interface, multiple steps were programmed in an operation cycle to prepare a single reaction mixture; 32 operation cycles were compiled in sequence to create the entire in situ click chemistry library for a given run. Figure 2 illustrates the four critical steps for the preparation of the reaction mixture, using the inhibitor-containing control reaction between acetylene **1** and azide **2** as an example. First, the metering pumps were used to introduce azide **2**, acetylene **1**, and inhibitor **22** into the mixing loop sequentially (flow rate $\approx$ 10 nL s^{-1}; Figure 2a). PBS solution was then employed to fill the round-shaped loop completely. Second, the reagent solutions were mixed for 15 seconds in the nL-level mixing loop (circulation rate $\approx$ 18 cycle min^{-1}; Figure 2b) using the mixing pumps. Third, PBS solution (flow rate $\approx$ 25 nL s^{-1}) was employed to introduce the 250-nL reagent mixture, along with 3.8 µL of the

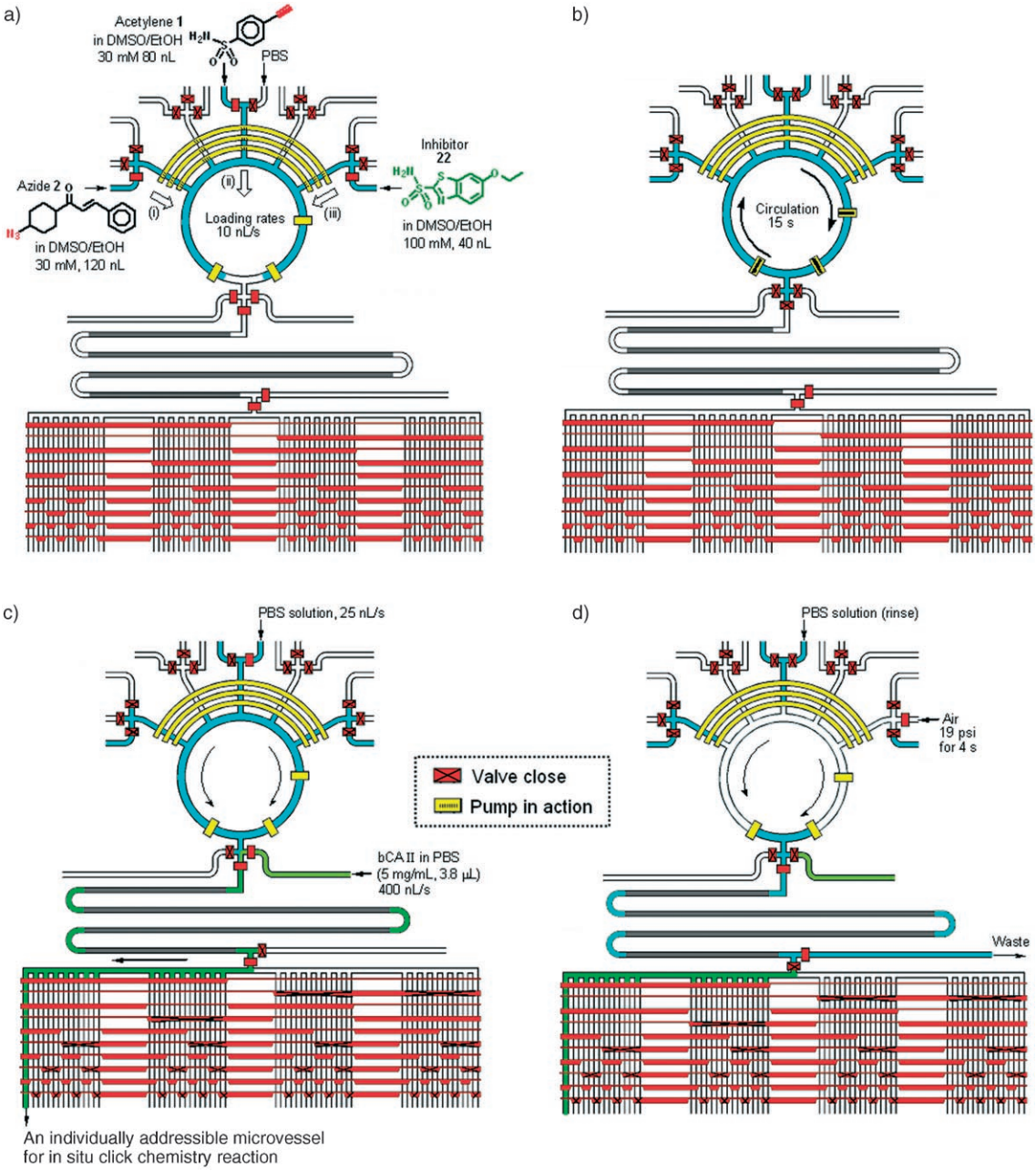
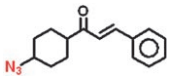
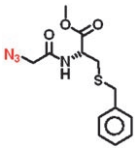
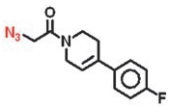
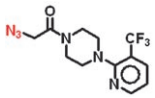
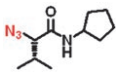
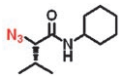
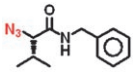
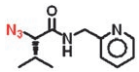
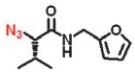
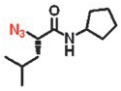
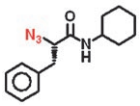
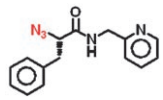
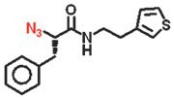
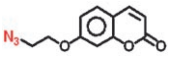
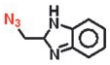
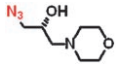
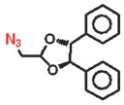
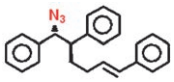
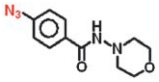
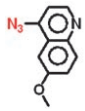


Figure 2. Schematic diagrams that illustrate the four sequential processes for preparing an individual in situ click chemistry mixture in the reaction circuit (this example describes a control experiment, which utilizes the acetylene **1**, azide **2**, and the active-site inhibitor **22**): a) Loading of the reagents in the mixing loop. b) Mixing of the reagent mixture. c) Introduction of the reagent mixture and enzyme into the chaotic mixture and final transportation via the multiplexer into the corresponding microvessel. d) Rinsing and drying of the microchannels.

bCAII solution (flow rate $\approx 400\ nL\,s^{-1}$), into the chaotic mixer (Figure 2c). The microfluidic multiplexer guided the resulting reaction mixture into the corresponding microvessel. Finally, the microchannels were rinsed with PBS solution (2 μL) and then purged with a flow of air to prevent cross-contamination (Figure 2d). The preparation of the entire screening library, which involved 32 operation cycles, took approximately 30 minutes (ca. $57\ s\,cycle^{-1}$). The chemical reaction circuit was then placed in a moisture-regulated incubator at 37 °C for 40 h to complete the reactions.

After incubation, the resulting reaction mixtures were collected from the respective microvessels. Each microvessel was rinsed with MeOH (5 μL × 3), and the rinsing solutions were combined with the original reaction mixtures. For reference purposes, the expected 1,4-disubstituted ("*anti*") triazoles were prepared separately from the corresponding Cu^{I}-catalyzed reactions.[21] The LC-MS analyses indicated that 10 out of the 20 reaction combinations had led to the formation of triazole products in the presence of bCAII. For comparison, all 20 in situ click chemistry reactions were also

Table 1: Summary of in situ click chemistry screening results between acetylene **1** and azides **2**–**21** obtained using the chemical reaction circuits.

Azide	Result[a]	Azide	Result[a]	Azide	Result[a]	Azide	Result[a]
 **2**	hit (hit)	 **3**	no hit (no hit)	 **4**	hit (hit)	 **5**	no hit (no hit)
 **6**	hit (hit)	 **7**	hit (hit)	 **8**	hit (hit)	 **9**	no hit (no hit)
 **10**	hit (hit)	 **11**	no hit (no hit)	 **12**	hit (hit)	 **13**	no hit (no hit)
 **14**	hit (hit)	 **15**	hit (hit)	 **16**	hit (hit)	 **17**	no hit (no hit)
 **18**	no hit (no hit)	 **19**	no hit (no hit)	 **20**	no hit (no hit)	 **21**	no hit (no hit)

[a] The results obtained for reactions performed in 96-well microtiter plates are indicated in parentheses.

performed in 96-well microtiter plates. The results of the in situ click chemistry screening between acetylene **1** and the 20 azides (**2**–**21**) in the new microfluidic format and the conventional 96-well system are summarized in Table 1 and reveal a very similar outcome.[21] LC-MS analyses of a positive hit identification obtained for the screening reaction between acetylene **1** and azide **2** and its control studies are illustrated in Figure 3, while those obtained for a negative hit identification between acetylene **1** and azide **3** are shown in Figure 4.

Previously, we reported the use of integrated microfluidics for sequential synthesis. [9] Here, we have utilized integrated microfluidics for parallel screening of an in situ click chemistry library. Our results suggest that integrated microfluidics promises to make lead discovery through in situ click chemistry more convenient and reliable, less expensive, and more diverse. Generally, most chemical reactions are performed either sequentially or in parallel. We believe that integrated microfluidics provides chemists a new playing field, where a wide range of chemical reactions can be performed beyond conventional settings, with

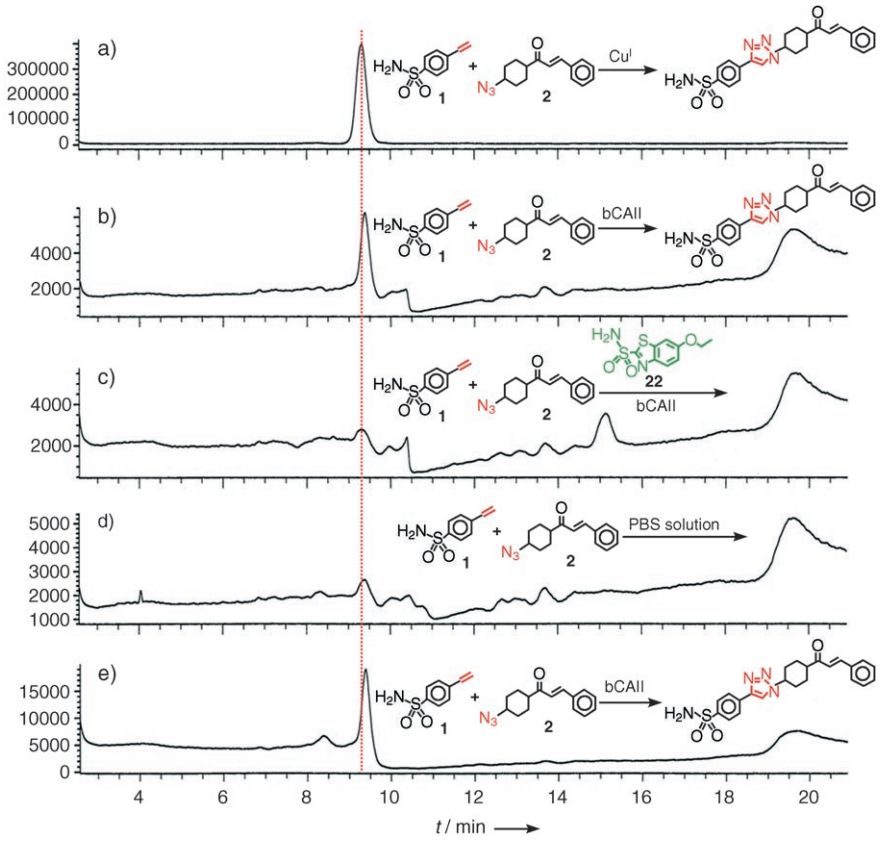


Figure 3. In situ click chemistry reactions between acetylene **1** and azide **2**, analyzed by LC-MS. The triazole product is indicated by the red dashed line. a) Triazole product obtained through Cu^{I}-catalyzed reaction (room temperature, 24 h). b–d) Microchip-based reactions performed at 37 °C for 40 h in the presence of bCAII (b), in the presence of both bCAII and inhibitor **22** (c), and in the absence of bCAII (d). e) Reaction performed at 37 °C for 40 h in a 96-well microtiter plate in the presence of bCAII.

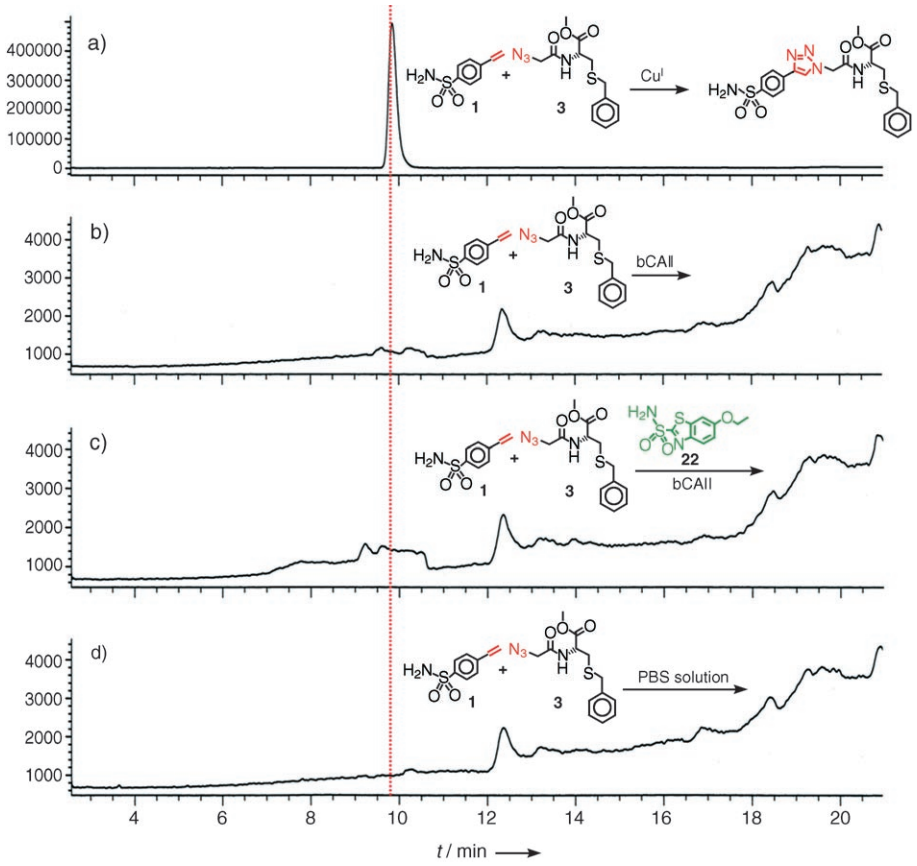


Figure 4. In situ click chemistry reactions between acetylene **1** and azide **3**, analyzed by LC-MS. The triazole product is indicated by the red dashed line. a) Triazole product. b–d) Microchip-based reactions performed at 37 °C for 40 h in the presence of bCAII (b), in the presence of both bCAII and inhibitor **22** (c), and in the absence of bCAII (d).

additional advantages of low reagent consumption, precise control over reaction conditions, faster reaction kinetics, cost efficiency, short design/fabrication processes, and rapid redesign/improvement cycles.

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