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A Synthetic Reaction Network: Chemical Amplification Using Nonequilibrium Autocatalytic Reactions Coupled in Time

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Abstract: This article reports a functional chemical reaction network synthesized in a microfluidic device. This chemical network performs chemical 5000-fold amplification and shows a threshold response. It operates in a feedforward manner in two stages: the output of the first stage becomes the input of the second stage. Each stage of amplification is performed by a reaction autocatalytic in Co^{2+}. The microfluidic network is used to maintain the two chemical reactions away from equilibrium and control the interactions between them in time. Time control is achieved as described previously (*Angew. Chem., Int. Ed.* **2003**, *42*, 768) by compartmentalizing the reaction mixture inside plugs which are aqueous droplets carried through a microchannel by an immiscible fluorinated fluid. Autocatalytic reaction displayed sensitivity to mixing; more rapid mixing corresponded to slower reaction rates. Synthetic chemical reaction networks may help understand the function of biochemical reaction networks, the goal of systems biology. They may also find practical applications. For example, the system described here may be used to detect visually, in a simple format, picoliter volumes of nanomolar concentrations of Co^{2+}, an environmental pollutant.

Introduction

In this article, we describe a synthetic chemical reaction network that performs a function: it uses autocatalysis in a time-controlled microfluidic device to perform one- and two-stage chemical amplification. We are interested in synthesizing functional chemical networks that mimic biochemical reaction networks. By a "functional chemical network" we refer to a set of interacting chemical reactions that is away from equilibrium and that can perform a function. Examples of functions performed by such networks include amplification, computation, and self-regulation. Common attributes of such a network are nonlinear kinetics, compartmentalization, and interactions among the reactions in space and time.[1−5] An example of a chemical network is an array of 16 coupled reactions that performed pattern recognition, a type of computation also common in neural networks of the brain.[1,2]

Synthesis is important in chemistry because it poses intellectual challenges and because it provides a starting point for new investigations. The synthetic targets span those inspired by nature[6] and those produced by abstract human thought.[7] Chemical synthesis is essential for generating and testing hypotheses in areas of science ranging from materials chemistry to catalysis to biological chemistry. Successes of chemical synthesis rely on the development of a conceptual framework and a set of laboratory tools and techniques that can be used to implement and test the concepts.

Synthesis of chemical networks requires development of new concepts. General thermodynamic theories that can be used to describe far-from-equilibrium systems are very important and are being developed.[8a] Large networks of biochemical reactions, with hundreds of kinetic parameters, would present a formidable computational challenge even if all of the parameters were known. Lack of exact theoretical description is not necessarily a problem for the development of effective synthetic strategies. Synthetic chemistry had been developing successfully before the Schrodinger equation could be solved with useful accuracy for the molecules of interest. The essence of the equation has been captured in approximate but very powerful concepts such as "chemical bond", "functional group", and "mechanism".[5] These physical organic concepts allow a chemist not to treat explicitly every atom in the molecule, but instead to simplify a complex molecule into a set of modules with known reactivity (functional groups), and then apply rules (mechanisms) to predict how these modules would interact. These concepts have been combined with approaches such as retrosynthetic analysis[8b] to create a framework for synthesis of organic molecules and even large biomolecules and supramolecular assemblies. Development of a conceptual framework of analogous power and simplicity to describe reaction networks would be important for both the synthesis of chemical reaction networks and for understanding

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10.1021/ja031689l CCC: $27.50 © 2004 American Chemical Society

of complex reaction networks in biology. We believe that the modular approach used in physical organic chemistry (functional groups as modules) may be extended to reaction networks.[5,9] In this approach, the mechanism by which the biochemical network functions is hypothesized, the network is separated into interacting modules, and then each module is represented by a single chemical reaction with appropriate kinetics. The network is then recreated using these chemical reactions (e.g., in a microfluidic device). If the hypothesis were correct, the simple chemical network must reproduce the function of the biochemical network by which it was inspired. This approach appears to work well for modeling hemostasis,[9] but it has to be tested on other biochemical systems and complementary approaches have to be investigated. Quantitative metrics used to describe biochemical modules would be important to develop, for example, in analogy to Hammett linear free-energy relations and substituent constants used to describe organic functional groups.[5]

Synthesis of chemical networks requires development of new experimental tools. Synthesis of chemical networks is challenging because it requires techniques for maintaining large numbers of chemical reactions away from equilibrium and for controlling interactions among them in space and time. These problems are not commonly encountered when molecules are synthesized. Microfluidics is attractive as a technique to solve such problems. For example, a self-regulating chemical system has been reported using pH-responsive hydrogels in a microfluidic device.[5,10] We have developed and used here a droplet-based microfluidic system implementing precise time control.[11] This system enables us to recreate the process of chemical amplification.

Experimental Details

Poly(dimethylsiloxane) (PDMS, Sylgard brand 184 silicone elastomer kit) was purchased from Dow Corning. 1*H*,1*H*,2*H*,2*H*-perfluoro-1-octanol (98%) and potassium peroxomonosulfate (Oxone) were obtained from Alfa Aesar. 2-(5-Bromo-2-pyridylazo)-5[*N*-*n*-propyl-*N*-(3-sulfopropyl)amino]phenol, disodium salt, dihydrate (5-Br-PAPS) was obtained from Dojindo Laboratories. Fluorocarbon 3283 was obtained from 3M. Fluorosurfactant Zonyl FSO 100 was obtained from DuPont. Oil used in the large microchannel was 3M fluorocarbon 3283: 1*H*,1*H*,2*H*,2*H*-perfluoro-1-octanol (98%) mixed 10:1 (v/v). Oil used in the small microchannel was 3M fluorocarbon 3283 saturated with FSO. Phosphate buffer (pH 7.0) was prepared from potassium dihydrogenphosphate. Cobalt(II) solutions were prepared by dissolving cobalt(II) chloride hexahydrate in water. The solution of Co(III)−5-Br-PAPS was prepared from 1×10^{-3} M 5-Br-PAPS and 5×10^{-4} M cobalt(II) at 70 °C. Once Co(III)−5-Br-PAPS complex was made, it was diluted to 7.5×10^{-5} M, and 5-Br-PAPS was added to be 4.5×10^{-5} M.

Microchannels were fabricated as previously described[12] using an SPI Plasma Prep II plasma cleaner. Microfluidic experiments were performed, and microphotographs were obtained as previously described.[12] Transition points for the reactions were determined by analyzing the microphotographs using ImageJ. The variations of transition times were due to variations in relative and absolute flow velocities. In each experiment, the number of microphotographs to show a transition at a particular time was called the frequency. Microsoft Excel and Igor Pro were used to perform data analysis.

Discussion

Chemical amplification is an essential function in signal transduction,[13] vision,[14] and metabolic regulation in biological systems.[15] Chemical amplification also has many practical applications. It is a key component of photography[16] and photolithography.[17] Polymerase chain reaction (PCR) is a widely employed method used to amplify specific nucleic acid sequences. We use the term "amplification" to describe a set of chemical reactions and transport processes by which a small input flux of molecules gives rise to a significantly larger output flux of molecules (where flux has units[18] of molecules $\times$ time^{-1} or moles $\times$ time^{-1}, in contrast to flux density[18] with units of molecules $\times$ time^{-1} $\times$ area^{-1}). We distinguish amplification processes occurring at different points in space and time. Autocatalytic reactions may be expected to be the most effective at amplification. They are involved in hemostatic response[19] and in apoptosis,[20,21] where rapid and large amplification is required. For example, caspases (cysteinyl aspartate-specific proteinases) use autocatalytic amplification to begin the process of programmed cell death.[20,21]

Amplification in hemostasis and apoptosis involves dozens of chemical reactions, and creating an exact chemical mimic of amplification in one of these systems would be difficult and probably would not provide new insights. Instead, we identified three features that are essential in an autocatalytic amplification network and asked whether these three features could be recreated using the minimal number of simple chemical reactions: (i) A nonequilibrium state, kinetically trapped in the absence of the autocatalyst; input flux of the autocatalyst triggers rapid production of the autocatalyst. (ii) A threshold response that makes this nonequilibrium state stable with respect to subthreshold flux of autocatalyst produced by biochemical fluctuations or by nonautocatalytic background reactions. This threshold may be set by inhibition, cleavage, or physical removal of the autocatalyst from the compartment.[22] (iii) Multiple stages of amplification, used to increase the overall amplification and also to allow for regulation and finer control of the threshold.

Single-Stage Amplification. To create a chemical network performing amplification, we used a reaction[23] that is autocatalytic in Co^{2+} ions (Scheme 1).

We used fluid flow in a microfluidic device (Figure 1) to create a nonequilibrium mixture of purple Co^{3+} complex (**1**) and $KHSO_5$ (**2**) by continuously supplying and mixing fresh

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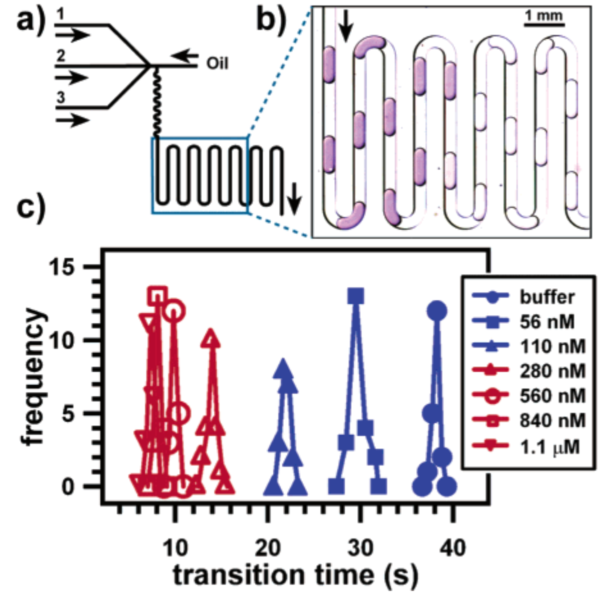
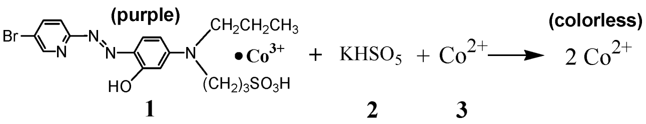


Figure 1. Single-step amplification. (a) A schematic of the microfluidic channels (315 × 315 μm^2 cross section). (b) A microphotograph of the microfluidic channel at the transition point of the autocatalytic reaction. (c) Distribution of transition times for various inputs of $[Co^{2+}]_o$. Transition times were determined using several microphotographs of the channel (similar to (b)). Frequency is defined as the number of microphotographs displaying a particular transition time. For example, a $[Co^{2+}]_o$ of 280 nM yielded 10 microphotographs with a transition time of 14 s. Aqueous concentrations of **1** and **2** were 25 μM and 0.030 M, respectively; $CoCl_2$ solution was prepared in 0.375 M KH_2PO_4 buffer, pH 7.0, and $[Co^{2+}]_o$ varied as specified in the legend. All solutions were diluted 3-fold when mixed inside plugs. Experiments were performed at 21.1−21.6 °C.

Scheme 1



reagents and removing products. This mixture decomposes autocatalytically in the presence of Co^{2+} to yield a colorless solution in which all of the Co^{3+} has been reduced (presumably by the decomposition products of the ligand) to Co^{2+}.[23] Because of autocatalysis, the majority (80%) of Co^{2+} is produced within 2.3 s at the end of a ~30 s reaction, leading to an abrupt color transition from purple to colorless (Figure 1b). The time at which the color transition occurs correlates directly to concentration of Co^{3+}.[23] The sharpness of the transition is due to the rapid conversion of Co^{3+} to Co^{2+} near the end of the autocatalytic reaction. We repeated the experiments described previously[23] using UV−vis spectrophotometry to confirm the sharp transition.

Time control was achieved by localizing the reagents inside water-in-fluorinated oil droplets (plugs) that were rapidly mixed by chaotic advection[24] and transported with no dispersion.[11] As expected, the reaction proceeded (in 37 s) even when buffer prepared in deionized water was used instead of the Co^{2+}-containing stream. The time required for the color transition to occur (transition time) decreased upon increasing $[Co^{2+}]_o$, the concentration of Co^{2+} in the reaction mixture at $t = 0$ s (Figure 1c). Transition times were obtained by dividing distance (from the point where plugs formed to the point at which the plug changed color) by flow velocity. The value of the flow velocity, calculated using the cross-sectional dimensions of the microchannel and the volumetric flow rate, agreed with the value obtained by direct measurement.

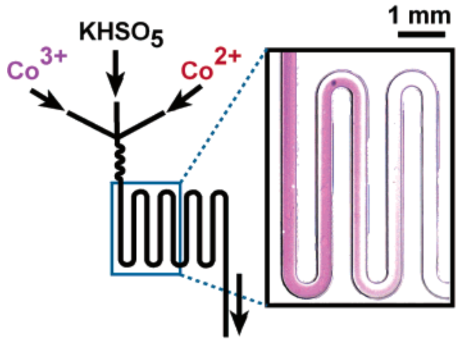


Figure 2. Single-stage amplification attempted in a laminar flow, without localizing reagents in plugs. Microphotograph of the microfluidic channel, showing a poorly defined transition.

We have reliably detected (that is, distinguished the reaction time from the 37 s reaction time of the buffer input) input $[Co^{2+}]_o = 56$ nM (reaction time = 29 s) using single-stage amplification. The concentration of Co^{2+} after the reaction equaled the initial concentration of the complex **1** (8.3 μM), corresponding to an amplification of up to 450-fold (flux of 6.6×10^{-15} mol Co^{2+}/s into the flux of 2.9×10^{-12} mol Co^{2+}/s). This level of amplification is similar to that in a single CMOS transistor (~100) or in a single biochemical step in G protein signaling (~100).

Time control achieved by compartmentalizing the reagents inside droplets is essential for this system to function. When droplets were not used to perform this reaction, the color transition was not well-defined because of slow mixing and large dispersion in laminar flow (Figure 2). The reaction proceeded more rapidly in the part of the channel with the high initial concentrations of Co^{2+} autocatalyst. The autocatalyst produced by the reaction diffused across the channel and initiated the reaction in other parts of the flowing reaction mixture.

Two-Stage Amplification. To increase the level of amplification and to generate a threshold response, we configured a microfluidic network for two-stage amplification. In this feed-forward network, two chemical reactions were coupled in a time-controlled manner. After a well-defined residence time, easily tuned by adjusting the length of the first channel and/or the flow velocity, the output of the first stage was fed as the input into the second stage (Figure 3). The first stage of amplification was performed in a channel with a small cross section, where each droplet was approximately 150 pL. Ten of these small droplets were merged[11] into each much larger droplet (~80 nL) of the second stage, performed in a larger channel. The difference in droplet sizes facilitated merging.[11]

Threshold response may be achieved by the competition of two reactions producing and removing the autocatalyst on two time scales.[22] In this network it is achieved (at the point of merging) by competition between the time scale of formation of the autocatalyst by the reaction and the time scale of removal of the autocatalyst by the flow. For example, in Figure 3, the residence time of the reaction mixture in the first channel (purposefully limited to 25 s by the channel length and flow velocity) establishes a threshold $[Co^{2+}]_o$ of 110 nM in the first section for which the second stage of amplification takes place. For all initial concentrations of Co^{2+} above this threshold,

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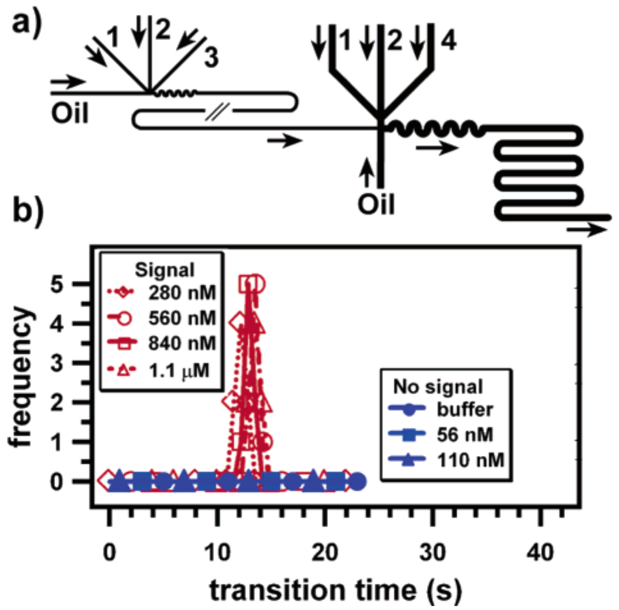

Figure 3. Two-stage amplification. (a) Schematic of the two-stage amplification device. Solutions 1, 2, and 3 are as defined in the caption of Figure 1; solution 4 was 0.375 M KH_2PO_4 buffer, pH = 7.0. The first stage takes place in the small 38 × 34 μm^2 channel, and the second stage in the large 315 × 315 μm^2 channel. (b) Distribution of transition times in the large section for varied $[Co^{2+}]_o$ input in the small section. Frequency is defined in Figure 1.

reaction in the first stage was finished (producing 8.3 μM Co^{2+} in the small droplets) by the time of merging. Merging of small droplets into large droplets produced initial $[Co^{2+}] = 210$ nM in the large droplets, corresponding to a reaction time of ~13 s ($[Co^{2+}]_o = 110$ nM input in the first section is diluted 3-fold to 37 nM when reagents are mixed inside a plug). For all $[Co^{2+}]_o$ below the threshold, reaction in the small section was incomplete and gave a reaction time over 30 s in the second stage. We monitored the second stage of the reaction from 2 to 23 s (limited by the length of the microfluidic channel) and observed the transition times shown in Figure 3b that correspond to the threshold response. The abruptness of the transition at the reaction endpoint and fine control of the interactions of the two reactions in time enabled us to create this threshold response. Overall, 5000-fold amplification was achieved, from 1.6 fmol/s to 7.6 pmol/s. This amplification was accomplished via an ~90-fold amplification in the first stage (1.6 fmol/s to 140 fmol/s) and an ~60-fold amplification in the second stage (140 fmol/s to 7.6 pmol/s). We have seen up to 450-fold amplification for $[Co^{2+}]_o = 56$ nM in a single-stage experiment (as discussed above). Setting the threshold concentration to $[Co^{2+}]_o = 110$ nM reduced the amplification in the first stage to 90-fold amplification.

Effects of Mixing. It is well-known that mixing affects the rates[25] and even outcomes[26] of autocatalytic reactions. Efficient mixing reduces the rates of autocatalytic reactions by removing pockets of locally high concentration of autocatalyst.[25] We have observed that this autocatalytic reaction is sensitive to the rate of mixing; the reaction in the small channel (mixing time ~0.02 s) consistently occurred more slowly than the same reaction in the large channel (mixing time ~0.5 s). We have good estimates for mixing time in these channels because we have previously characterized the scaling of mixing by chaotic advection as a function of the cross-sectional dimension of the channel.[27] We confirmed this observation by performing mixing experiments both in microfluidic channels and in a vial on a benchtop (two sets of experiments are shown in Figure 4). We did not characterize mixing time in a vial, but assumed that it correlated with the inverse of the stirring rate.

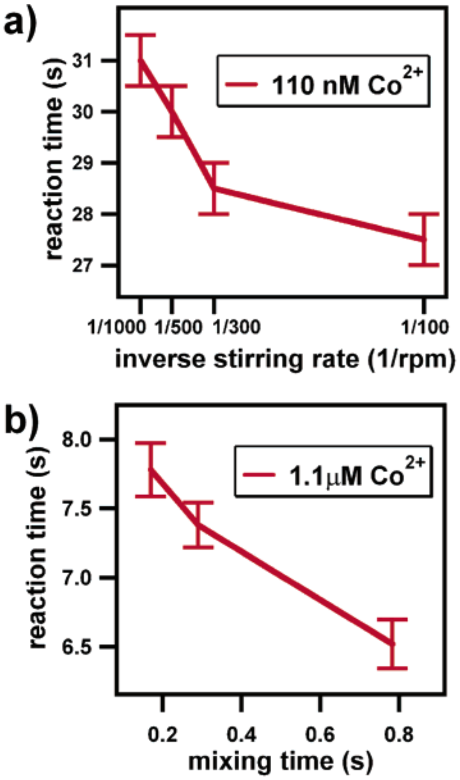

Figure 4. Effect of mixing. Reaction time as a function of the mixing time for the autocatalytic reaction shown in Scheme 1. Input $[Co^{2+}]_o$ is shown in the legend. (a) Reaction conducted on 0.9 mL scale in a vial; inverse mixing rate is plotted instead of mixing time. (b) Reaction performed in 80 nL droplets in a microchannel.

This effect is also evident when comparing the data from one- and two-stage amplification. In the single-stage experiment (315 × 315 μm^2 channel; mixing time ~0.5 s), the reaction time for 110 nM $[Co^{2+}]_o$ is ~22 s. This time should be sufficiently short to yield a signal in the two-stage experiment, which has a 25 s threshold. However, the mixing time in the smaller (38 × 34 μm^2) channel of the two-stage experiment is much lower (~0.02 s). Rapid mixing in the small channel extends the reaction time for 110 nM $[Co^{2+}]_o$ from 22 s to over 25 s, keeping it below the threshold of the two-stage system.

Conclusion

We have shown that microfluidics can be used to maintain two chemical reactions away from equilibrium and control their interactions in time, leading to a chemical network that performs a simple function: two-stage chemical amplification with a threshold response. Time control was essential: when laminar flows were used instead of droplets, reactions were not compartmentalized and transition times were ill-defined. The two-stage, 5000-fold amplification in this system is comparable to typical two-stage amplification in a transistor (~10 000-fold),

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but is significantly lower than the multistage photon/rhodopsin → tranducin → cGMP amplification in human vision (∼100 000-fold). This microfluidic system requires small amounts of reagents and could be operated in an autonomous mode with low power consumption. It may be useful for environmental monitoring of heavy metal pollutants; other reactions that use other environmental pollutants (heavy metals[28,29] and oxyhalides[30−33]) as autocatalysts are known. These amplifying systems could be particularly useful in conjunction with microfluidic logic circuits and self-regulating systems.[10,34]

We have synthesized a functional chemical network by identifying the essential components of the biochemical amplification networks, and generating a simple chemical mimic that performs the same function: chemical amplification. This chemical network uses only two coupled reactions and performs a rather simple function. The microfluidic system used here provides time control on time scales from milliseconds to hundreds of seconds[11,24] and even to days,[35] and we expect that such an amplifying network may be synthesized from other autocatalytic reactions[19,20,36] occurring on these time scales. We are using a modular approach, inspired by physical organic chemistry and similar to the one described here, to understand and chemically mimic the biochemical network of hemostasis performing a more sophisticated function of self-repair.[9] We believe that it is important to develop a systematic conceptual and experimental approach to the synthesis of larger reaction networks performing more sophisticated functions. It remains to be seen if an approach conceptually similar to the one used to synthesize organic molecules is successful. Synthetic functional chemical networks could find practical applications and may help understand the design principles of functional network of biochemical reactions.

Acknowledgment. This work was supported by Office of Naval Research Young Investigator Award (N00014-03-10482). At The University of Chicago, work was performed at the MRSEC microfluidic facility funded by NSF. Photolithography was performed at MAL of UIC. We thank Yelena Koldobskaya for insightful comments.

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