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Continuous Monitoring of Enzyme Reactions on a Microchip: Application to Catalytic RNA Self-Cleavage

Tracy L. Paxon,[†] Trevor S. Brown,[†,‡] Hsiao-yu Nancy Lin,[†] Sam J. Brancato,[†] Elizabeth S. Roddy,[†] Philip C. Bevilacqua,[†,‡] and Andrew G. Ewing*[,†,‡]

Department of Chemistry and The Huck Institutes of the Life Sciences, The Pennsylvania State University, University Park, Pennsylvania 16802

Kinetic analysis of RNA enzymes, or ribozymes, typically involves the tedious process of collecting and quenching reaction time points and then fractionating by polyacrylamide gel electrophoresis (PAGE). As a way to automate and simplify this process, continuous analysis of a ribozyme reaction is demonstrated here using completely automated capillary sample introduction onto a microfabricated device with laser-induced fluorescence detection. The method of injection is extremely reproducible thereby standardizing data analysis. A 30-nucleotide ribozyme model, the self-cleaving lead-dependent ribozyme, or "leadzyme", which cleaves into a 24-mer and a 6-mer in the presence of Pb^{2+}, was end-labeled with fluorescein (FAM) and used to demonstrate the potential of this technique. After manually initiating the cleavage reaction by Pb^{2+} addition, reaction samples were automatically injected directly into the parallel separation lanes of the chip via a capillary at predetermined time intervals, thus eliminating the need for additional sample-handling steps. The FAM-labeled leadzyme starting material and products were monitored for 60 min in order to ascertain kinetic information. The effect of lead acetate concentration on cleavage rates was also studied, and the results are in agreement with rates determined by conventional hand-mixing/PAGE analysis. This work demonstrates, through the use of a simple ribozyme model, the potential of this method to provide valuable kinetic information for other, more complex, biologically relevant RNA and protein enzymes.

In 1982, the first catalytic RNA, or ribozyme, was reported.[1] Other natural ribozymes were soon discovered,[2] and beginning in the early 1990s, nonnatural ribozymes were identified through in vitro selection, a process by which individual RNA molecules that perform a specified function are isolated from a library of 10^{13}−10^{15} sequence variants and amplified.[3−7] RNA serves a variety of functions in the body including the mobilization and interpretation of genetic material. Since ribozymes behave similarly to protein-based enzymes, there is heightened interest in the discovery of new ribozymes. Recently, new drug therapies have employed ribozymes in the treatment of disorders ranging from infectious diseases to cancer.[8] The applications of these RNA catalysts can be expanded as kinetic information is ascertained. These kinetic studies should provide the basis for understanding the mechanism of cleavage, thus furthering our fundamental knowledge of these important compounds.

The most common method for assaying ribozyme kinetics has been based upon polyacrylamide gel electrophoresis (PAGE). This assay involves removing aliquots from a cleavage reaction at a series of time points, quenching, and separating them in parallel on a slab gel. Unfortunately, this method is very labor intensive and typically requires the tedious collection of samples at predetermined time points such that samples are stagnant for the duration of the experiment until separation and analysis can be performed. This method is time-consuming, costly, and typically involves the use of radiography for detection, though fluorescence is sometimes employed. In some instances, HPLC has been used to quantify amounts of ribozyme substrate and cleavage fragments.[9] However, HPLC does not offer high throughput or continuous analysis of a cleavage reaction. Alternatively, the cleavage and folding kinetics of various ribozyme reactions have been monitored in real time through base-specific quenching or enhancement of labeled substrates.[10,11] The drawback to these fluorescence methods is the inability to directly relate a change in fluorescence intensity to bond cleavage and the possibility that bond cleavage might be spectroscopically silent. Other spectroscopic methods such as fluorescence resonance energy transfer (FRET) have been applied to RNA systems and have provided

* To whom correspondence should be addressed. E-mail: age@psu.edu. Fax: (814) 863-8081. Tel: (814) 863−4653.

[†] Department of Chemistry.

[‡] The Huck Institutes of the Life Sciences.

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kinetic information on the hammerhead ribozyme.[12−14] However, FRET, in addition to suffering from problems similar to the other techniques, requires the use of two fluorescent tags and, therefore, two modifications of the RNA.

Capillary electrophoresis (CE) with automated capillary sample introduction onto a multichannel microchip is an ideal technique for continuous monitoring of enzyme reactions, specifically self-cleavage by a catalytic RNA. Conventional CE provides fast separation times and small sample requirements and has been reviewed extensively for a variety of applications;[15] however, conventional CE is a serial technique, which significantly limits throughput. Capillary array electrophoresis, in which separations are performed on an array of parallel silica capillaries, was introduced by Mathies[16] and subsequently used to perform high-speed, high-throughput DNA sequencing[17,18] and DNA fragment sizing.[19] The miniaturization of capillary electrophoresis onto a microchip was realized and emphasized its ability to increase performance by reducing analysis times and reagent volumes.[20−23] Since then, extensive work has been performed in microchips for the analysis of a variety of substances including amino acids,[24] small drug molecules,[25] peptides,[26] oligonucleotides,[27] proteins,[28,29] and DNA fragments.[30] Initial microchip separation devices consisted of a single separation channel; however, recent developments take advantage of the ability to multiplex separation channels on microchips thereby providing a means to achieve high-throughput separations.[30−39] The ability to multiplex channels on a microchip coupled to capillary sample introduction for the analysis of DNA has been previously demonstrated in our group.[40,41]

In this article, we describe experiments that provide a novel application of chip electrophoresis with capillary sample introduction. We present an enhanced separations-based method for analysis of ribozyme cleavage reaction kinetics. An automated capillary sampling device has been used to transfer sample from a single reaction vial to a microfabricated chip with an array of separation lanes. Previous work has employed electrokinetic injection via hand-toggling a power supply for the analysis of discrete plugs of sample[41] and methods to monitor the progression of a protein enzyme catalyzed DNA digest in real time utilizing pressure injections performed by toggling a manual valve apparatus.[40] For the first time, this injection method has been completely automated through the integration of an air-actuated valve, providing extremely reproducible sample injections and ultimately more consistent data. In addition, this methodology has been applied to the study of RNA enzyme reactions. The reaction kinetics and divalent ion dependence of the self-cleaving, lead-dependent ribozyme have been examined.

EXPERIMENTAL SECTION

Microfabrication. The separation devices were fabricated using traditional lithography techniques at the Penn State Nanofabrication facility as described previously.[41] Borofloat glass (Technical Glass Products, Mentor, OH) was used to make the chips for these experiments. Each chip consisted of five parallel channels, 500 μm wide, 250 μm deep, spaced 5 mm apart. The top plate was etched with the channels and subsequently thermally bonded to the bottom plate of the same dimensions in a programmable oven (Vulcan 3-3130, Ney Dental, Inc., Bloomfield, CT). The temperature was ramped to 650 °C, held at that temperature for 4 h, and then gradually cooled to room temperature. With the bonding of the top plate, a chip with end-on access to the separations lanes was created.

Sieving Matrix Preparation. The bonded chips were filled with linear polyacrylamide (LPA) solutions using a water aspirator system. The 3.8% (w/v) LPA solution was prepared by dissolving acrylamide powder (ICN Biomedicals, Aurora, OH) in 30 mM tris−borate buffer containing both 15 mM EDTA and 7 M urea for metal chelation and denaturing purposes, respectively. Polymerization was initiated with 10% ammonium persulfate (Aldrich, Milwaukee, WI) and catalyzed with *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (ICN Biomedicals). After degassing the LPA, the chip was filled and suspended between two buffer reservoirs containing the same solution as the chip.

Instrumentation. The automated capillary sample introduction and detection system have been described previously.[41] Briefly, an argon ion laser (Coherent, Santa Clara, CA) was used as an excitation source for laser-induced fluorescence detection. The beam was sent through a series of optics to shape it into a line that was focused across the width of the chip approximately

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6−6.5 cm from the point of injection. A power supply (Bertan, Hicksville, NY) was used to apply 1.0 kV across the chip for electrophoresis. The 5′-fluorescein (FAM)-labeled precursor leadzyme 30-mer and labeled 5′ product 6-mer were deposited into the LPA-filled lanes via the capillary, separated, and excited as they passed through the laser line. The resulting fluorescence was collected with a liquid nitrogen-cooled CCD camera (Photometrics, Tuscon, AZ) at a rate of one exposure/s. Data were collected using MAPS 2.0 software (Photometrics) and viewed using Transform 3.4 (Fortner Software LLC, Sterling, VA). Analysis was performed using Excel (Microsoft Corp., Redmond, WA) and KaleidaGraph (Synergy Software, Reading, PA). A fused-silica capillary (50-μm inner diameter, 150-μm outer diameter, ∼45 cm in length, Polymicro Technologies, Phoenix, AZ) was used to transfer sample from the reaction vial into each of the lanes.

Automated Injection Method. A six-port air actuated valve (Valco Instruments Co. Inc., Houston, TX) was integrated into the injection system to provide reproducible pressure injections of sample through the capillary and into the chip. A glass vial containing the sample was pressurized with nitrogen gas to an appropriate pressure via the air actuated valve for 2 s to conduct an injection. The capillary was inserted into the vial through a Teflon tee, which held a septum, so that the tip was submerged in the sample. A Labview (National Instruments, Austin, TX) program, written in-house, was used to close the valve, causing an injection of sample into a lane on the chip. The Labview programs and CCD camera were started immediately after the capillary was pressurized. The entire process, from the addition of the initiating reagent, lead acetate, to the first injection into the chip could generally be accomplished in 53 s.

Manipulator. A computer-controlled micromanipulator (SD Instruments, Inc., Grants Pass, OR) was used to move the sample introduction capillary from one lane to another across the chip, as previously described.[41] Initial alignment of the capillary with the first lane was achieved by manual adjustment of the manipulator. A Labview (National Instruments) program, written in-house, was used to insert the tip of the capillary into each lane on the chip. While the capillary was inside the lane, the valve was pressurized for 2 s to inject a small plug of sample. The manipulator was programmed to insert the capillary into all five lanes and return it to the starting position. The maximum velocity used in these experiments enabled the manipulator to make one injection/14 s into each of the five lanes. After injection into the fifth lane, the manipulator was returned to the starting position. This process was repeated as many times as necessary. To avoid overlap of separations in each lane, the capillary remained at the first lane for an additional 2−5 min before restarting the Labview program. The manipulator and injection programs were coupled together such that as the manipulator placed the capillary tip into a separation lane a pressure injection occurred simultaneously.

RNA Cleavage Reactions. RNA was obtained from Dharmacon, Inc. (Lafayette, CO), and was 5′-end labeled with FAM (λ_{ex}= 494 nm, λ_{em}= 525 nm). The sequence of the strand was as follows: 5′-(FAM)GCGACC^GAGCCAGCGAAAGUUGGGAGUCGC-3′, where (^) represents the site of self-cleavage. The RNA was deprotected according to the manufacturer's instructions. The sample was reconstituted in 15 mM MOPS (Sigma, St. Louis, MO), pH 7.0 at 25 °C, to a concentration of 0.75 mM as determined spectrophotometrically.[42,43] The RNA was diluted to 4.4 μM in 15 mM MOPS and frozen in 50-μL aliquots. Before use, the RNA was renatured by heating to 90 °C for 2 min and cooling to room temperature (25 °C) on the benchtop for 10 min. A 10× lead acetate solution (2 μL) was then added to 18 μL of this RNA solution that was prepared in a small centrifuge tube placed inside the glass vial to be pressurized. Reaction temperature was room temperature (∼25 °C) in these experiments.

Traditional Gel Electrophoresis. For the purpose of collecting data by traditional PAGE assays, reactions were initiated in a similar fashion by the addition of 2 μL of a 10× lead acetate solution to 18 μL of a 4 μM RNA solution in 15 mM MOPS (Sigma), pH 7.0 at 25 °C. The RNA was renatured as above before use. Reaction samples (2-μL aliquots) were removed at predetermined times and quenched by mixing with an equal volume of formamide loading buffer containing 40 mM EDTA. These samples were immediately frozen by submerging in dry ice. After completing time point collection, the samples were allowed to thaw at room temperature before loading and fractionating a 2-μL sample of each on 10% polyacrylamide/8.3 M urea/1× Tris−boric acid−EDTA (TBE) gels. The gels were then dried and quantified using a Typhoon imager (Amersham Biosciences, Inc.) set to fluorescence mode, 400 V, normal sensitivity, with the 526 short-pass filter and green (532 nm) laser line excitation. Resulting band intensities were quantitated using ImageQuant software (Amersham Biosciences, Inc.). KaleidaGraph (Synergy Software) was used to plot and fit data.

Data Analysis. Cleavage-reaction data were fit with eq 1 (KaleidaGraph, Synergy Software),

$$f_{*P} = *P/*SM_0 = A + B\exp(-k_{obs}t) \quad (1)$$

where f_{*P} is the fraction of total leadzyme cleaved, A is the extent of reaction, B is a term that accounts for any burst phase by the relationship $f_{burst} = A + B$, k_{obs} is the observed first-order rate constant for leadzyme cleavage, and t is time. By plotting fraction cleaved versus time, the data could be fit to the single-exponential equation. Equation 1 is derived from the following simple reaction model,

$$*SM \rightarrow *P_1 + P_2$$

where * represents the 5′-FAM label, SM is starting material leadzyme, P_1 is the 5′-product 6-mer, and P_2 is the 3′-product 24-mer.

RESULTS AND DISCUSSION

Leadzyme Overview. An RNA molecule that undergoes autolytic cleavage with Pb^{2+}, the leadzyme, was previously isolated by an in vitro selection method.[44−49] The small size, excellent

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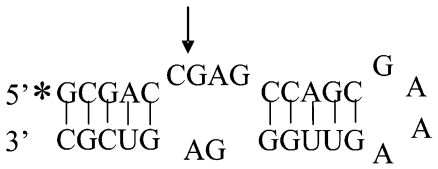

Figure 1. The 30-nucleotide leadzyme secondary structure.[44] RNA is 5′-labeled with fluorescein (*) and self-cleaves in the presence of Pb^{2+} to afford labeled 6-mer and unlabeled 24-mer products. The cleavage site is indicated by the arrow.

reactivity at room temperature, ease of labeling, and easily manipulated rates led to the choice of the leadzyme as an ideal model system to demonstrate the capabilities of our separation technique to probe RNA enzyme kinetics. These experiments utilized a small 30-nucleotide one-piece version of the leadzyme that exhibits self-cleavage 6 bases from its 5′ end (Figure 1). In the presence of Pb^{2+}, the leadzyme self-cleaves between C6 and G7 due to in-line attack by the 2′-OH of C6, which is facilitated by Pb^{2+}. The design allowed detection of intact leadzyme starting material (30-mer) and 5′-cleavage product (6-mer) by fluorescence. As the reaction continued, a decrease in 30-mer concentration was observed coinciding with an increase in 6-mer (see below).

Importantly, the leadzyme cleavage rate is sensitive to Pb^{2+} concentration, and therefore, kinetic rates could be easily controlled and tuned to the optimal values for our apparatus.[49]

Capillary Sample Introduction for Kinetic Assays of RNA Self-Cleavage on Chips. Capillary sample introduction has been used here as an effective tool to elucidate kinetic information from a ribozyme sample, specifically the leadzyme. A unique advantage of this technique is that the sampling device is independent of the separation device. Therefore, the separation of a sample at one time point does not have to be completed to start another, and an ongoing reaction can be sampled at different times. Consequently, faster sampling and a more continuous analysis of the cleavage reaction is accomplished.

A 5′-fluorescently labeled autocatalytic RNA known as the leadzyme has been chosen to demonstrate the capability of this separations-based system to monitor the progress of self-cleaving RNA reactions. Leadzyme cleavage was initiated and the mixture injected directly into the chip, eliminating the need for any sample-handling steps after addition of Pb^{2+} to the reaction vial. In this method, the capillary acts as a transfer device, carrying sample from an ongoing reaction to a microfabricated chip. As the sample enters the chip, the reaction is quenched on-line due to the presence of excess EDTA (15 mM), which chelates the Pb^{2+} (2−200 μM) required for cleavage. The efficiency of the quench is verified by the fact that curve fitting to a single exponential gives a trace that goes through the origin. Therefore, each injection into the chip captures a time point during the reaction, and both the labeled 6-mer and the labeled 30-mer sequences can be observed. The products of the leadzyme cleavage reaction were continuously monitored for 60 min. The fraction of leadzyme cleaved with time was plotted and fit to eq 1 and compared to rate constants from traditional hand-collected PAGE experiments.

The output from a leadzyme cleavage reaction in the presence of 2 μM lead acetate measured with capillary sample introduction and chip separation is illustrated in Figure 2. Figure 2A is a reconstructed plot of the fluorescent signal collected by the CCD camera during the course of the experiment. The spatial resolution of the chip is represented by pixel number and is retained in these images, allowing data to be collected from all five lanes simultaneously. The leadzyme reaction is initiated at time 0, and "injection time" reported refers to the length of time between initiation and when the sample moves into the channel. Once inside the channel, the sample must migrate to the detection beam, which takes a certain amount of time. The length of time the sample spends in the channel plus the injection time defines "separation time". Data illustrated in Figure 2A have been obtained by depositing the reacting leadzyme from the sample-filled capillary into each lane beginning at the far right (lane 1). The manipulator moves from right to left, depositing sample and returning to repeat the process after injecting into lane 5. The first, sixth, eleventh, and so forth, injections can therefore be represented by taking a cross section of a single separation lane. The injections are staggered in time due to constant chip potential throughout the experiment. Upon injection, analyte plugs begin migrating toward the laser line for detection. In this experiment, sample injections began 68 s after the reaction was initiated. The manipulator deposited sample every 14 s across the five lanes and paused for 300 s before repeating to ensure that separations do not overlap in a given lane. The manipulator is moved across the chip 16 times, which produces 80 separations in ∼85 min. Decreasing the pause time between injection sweeps or increasing the number of channels would increase throughput for the analysis of faster reactions. Figure 2B shows an expanded view of a single separation as highlighted by the box in Figure 2A. The top band marked with an asterisk represents the 6-mer cleavage product, while the lower band represents uncleaved 30-mer.

The electropherogram resulting from lane 3 of Figure 2A is depicted as a cross section of intensity versus time in Figure 2C. A clear change in sample composition is observed as the reaction progresses. The initial peak corresponds to the 30-base leadzyme starting material. In the presence of Pb^{2+}, the leadzyme is cleaved and the 30-mer peak diminishes over time, as the smaller, faster migrating 6-mer peak marked with an asterisk increases. Slight variation in total peak intensity is observed in Figure 2C due to manual injections that were used in this experiment only. Initial studies herein were carried out using the manual injection scheme presented by Roddy et al.;[40] however, current studies indicate less peak variability with automated injections (see below) and all subsequent data were collected using automated injections. The variation in this particular experiment is not a problem because all data analysis is performed by taking peak height ratios of product versus total RNA.

On-Line Measurement of Accelerated Leadzyme Self-Cleavage Reaction. Next, an experiment using automated capillary sample introduction into a microfabricated chip was performed using a higher, 10 μM, Pb^{2+} concentration to accelerate the rate and examine the Pb^{2+} dependence of the reaction (Figure 3). Under these conditions, the leadzyme reached completion in only 60 min (Figure 3A). Again, the first peak in each doublet set of peaks represents the *6-mer concentration while the second

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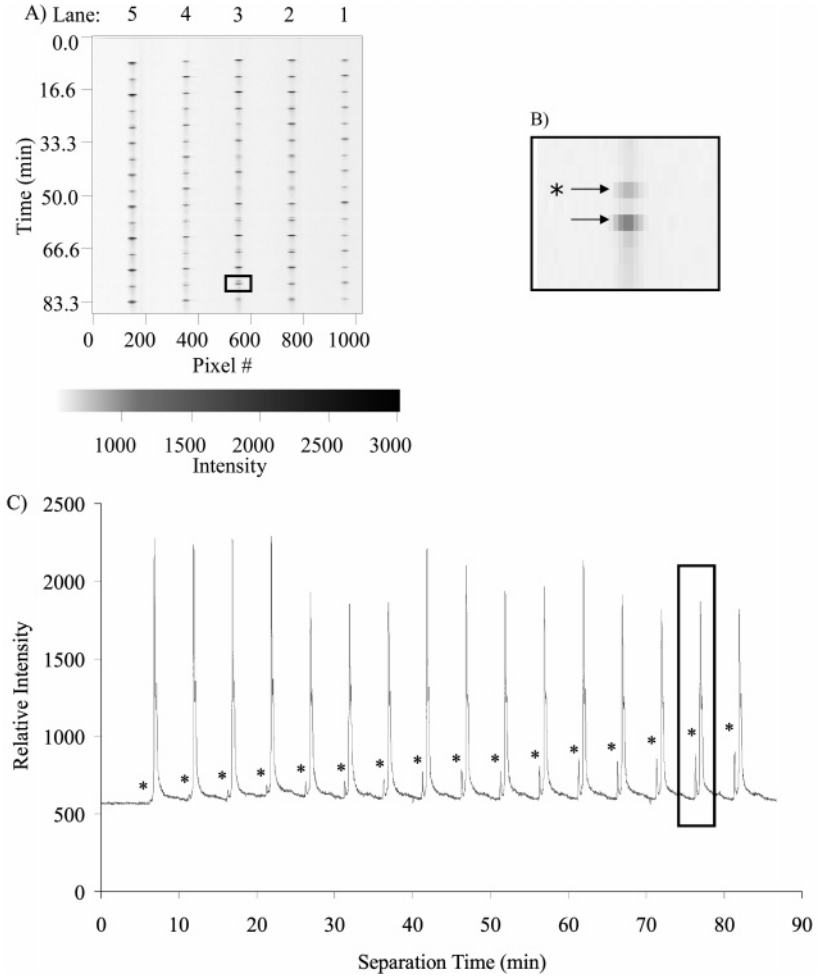

Figure 2. (A) Reconstructed plot of a 2 μM lead acetate cleavage reaction with manual injections illustrating separation time vs pixel number across the width of the chip. The shaded bar represents fluorescence intensity collected by the CCD. Sampling capillary was moved from right (lane 1) to left (lane 5) depositing sample in each channel and returning to lane 1 to repeat the process. Separation conditions: 3.8% LPA, 30 mM tris−borate, 15 mM EDTA, and 7 M urea, 1.0-kV separation potential, and ~6-cm separation distance. (B) Expanded view of a single separation as highlighted by the box in (A). Asterisk highlights 5′-product (6-mer). (C) Cross section through lane 3 of (A) illustrating intensity vs separation time of the cleavage reaction. Asterisk highlights 5′-product (6-mer) intensity increasing over time. Box corresponds to highlighted boxes in (A) and (B).

peak is indicative of the 30-mer concentration. Raw data (Figure 3A) were plotted as peak height versus injection time in Figure 3B. Product formation and starting material cleavage show mirror image behavior. The presence of only two peaks is consistent with a simple cleavage reaction, as well as the absence of side reactions, RNA degradation, and alternative conformations.

Data from this 10 μM Pb^{2+} reaction lead to a faster rate constant ($k = 0.045$ min^{-1}) and higher extent of reaction (76%) (Figure 3C) than 2 μM Pb^{2+} ($k = 0.03$ min^{-1} and <20%). A larger number of data points were intentionally collected at the beginning of the reaction due to the exponential nature of the process. Some variability is observed as all of the data points do not fall exactly on the curve and may be attributed to slight variations in the surface characteristics of each channel.

Channel-to-Channel Reproducibility with Automated Injection via the Capillary Interface. With the integration of an air-actuated valve for automated capillary sample introduction, a significant improvement in channel-to-channel and injection-to-injection reproducibility is observed for all concentrations examined. When standard injections of unreacted leadzyme were performed using the automated capillary sample introduction, peak heights varied by less than 1.9 ± 0.5%. This is much smaller than the 4.3 ± 3.0% variation observed when injections were performed by toggling a manual valve (data not shown).

Figure 4A shows the reconstructed plot of the first 30 min of a leadzyme cleavage reaction performed in the presence of an even higher concentration of Pb^{2+} (100 μM). Pressure injections were performed in each lane as described using the automated injection scheme. Electropherograms from lanes 1 and 2 consisting of the first 30 min (4 injections/channel) of the reaction are shown in Figure 4B. It is clear from these two sample traces that excellent channel-to-channel reproducibility has been achieved, although a slight difference in resolution is noticed. We believe this resolution difference is due to slight differences in surface characteristics of each channel as a result of wet etching during fabrication. For 100 μM Pb^{2+}, the reaction is sufficiently fast that it reaches ~50% cleavage before the second set of injections can be made.

It is important to examine all of the channels when determining fast reaction kinetics in order to obtain the maximum number of data points prior to 50% cleavage. We show that the increased channel-to-channel reproducibility makes it possible to examine

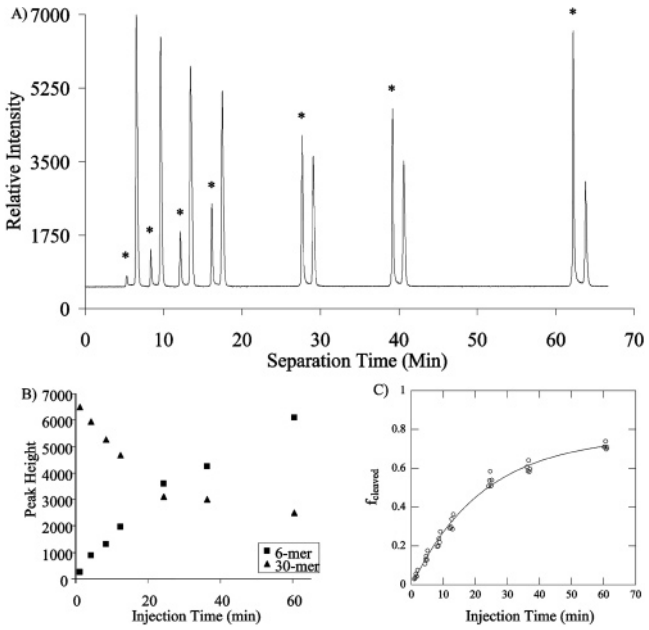

Figure 3. (A) Cross section through lane 2 of a leadzyme reaction in the presence of 10 μM lead acetate illustrating fluorescence intensity vs separation time. Asterisk highlights 5′-product (6-mer). Separation conditions same as Figure 2. (B) Plot of peak height for the 6- and 30-mer strands vs injection time for the experiment shown in (A). (C) Plot of fraction cleaved vs time of injection. Peak heights have been used to obtain the fraction cleaved by dividing the 6-mer by the sum of 6- and 30-mer. Each time the injection process is repeated, an additional five consecutive time points are collected; thus, a cluster of points are observed. Each cluster of five data points represents five consecutive injections as the capillary makes a single pass across the five individual lanes depositing sample in each lane as it moves. Values for the kinetic fit equation: $A = 0.76$; $B = -0.77$; $k_{obs} = 0.045$ min^{-1}; $t_{1/2} = 15.4$ min. All injections were monitored with an external clock, and the first set of peaks correspond to an injection made 53 s after the reaction was initiated. Subsequent injections were made at 4, 8, 12, 24, 36, and 60 min.

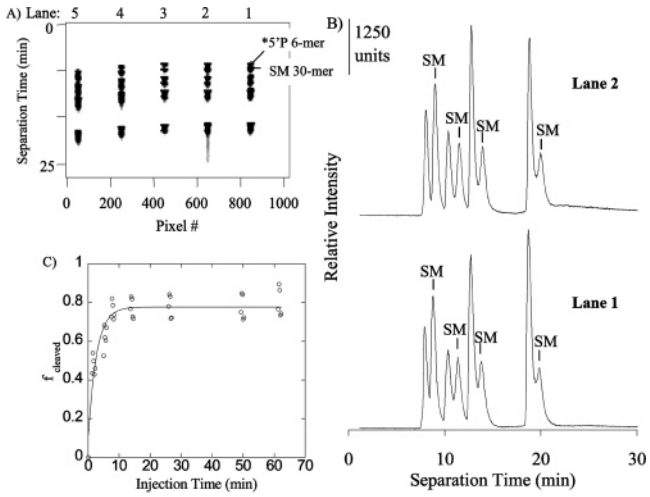

Figure 4. (A) Reconstructed plot of time vs pixel number across the width of the chip. The capillary was moved from right (lane 1) to left (lane 5) depositing sample into each channel individually. (B) Raw data from separation lanes 1 (bottom) and 2 (top) of a leadzyme cleavage reaction in the presence of 100 μM lead acetate. SM peak highlights the depletion of 30-mer starting material as the reaction progresses. (C) Plot of fraction leadzyme cleaved vs injection time, which shows that 50% cleavage is reached by the time of the second injection. The 0 time point was obtained by running the leadzyme reaction in the absence of Pb^{2+} (electropherogram not shown). Separation conditions are the same as Figures 2 and 3. Values for the kinetic fit equation: $A = 0.78$; $B = -0.60$; $k_{obs} = 0.37$ min^{-1}; $t_{1/2} = 1.9$ min.

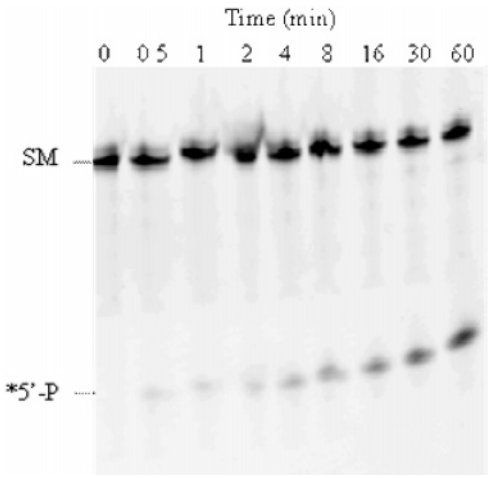

Figure 5. Hand-collected time points for 2 μM Pb^{2+} cleavage reaction separations with 10% PAGE/1× TBE/7 M urea. Uncleaved, starting material (SM, upper band) and 5′-cleavage product (*5′-P, lower band) were visualized and digitized using a Typhoon imager and quantitated using ImageQuant software (Molecular Dynamics). Time of the data point collection is provided across the top of the gel.

and compare data from different channels. Although comparing separate channels is not as critical for these leadzyme experiments because we can ratio the two peaks within a single injection, channel-to-channel reproducibility becomes increasingly important in experiments where a single peak is observed and peak ratios are not available. Focusing on the first four injections in each lane allows one to examine the cleavage reaction as it progresses. The reaction was initiated before the first injection (lane 1) was made; therefore, the cleavage reaction is already in progress. In Figure 4B, the faster moving cleaved 6-mer is the first peak to elute, while the uncleaved starting material (SM) leadzyme elutes immediately following. Comparing the first injection (lane 1) to the second injection (lane 2), a slight increase in 6-mer (peak 1) and a corresponding decrease in 30-mer (peak 2) is observed (Figure 4B). The second set of peaks shown (lane 1) represent the sixth injection. By the time this injection was made, the reaction had proceeded to ~52% cleavage (Figure 4C). After only 25 min, this reaction reached completion with ~78% of the initial sample cleaved as shown by the leveling off of the data in Figure 4C.

Comparison of the Chip Array to Traditional Electrophoresis. Reactivity at each concentration of Pb^{2+} was also examined by traditional gel electrophoresis for comparison and validation of the new automated method. First the 2 μM Pb^{2+} leadzyme reaction was examined (Figure 5) for comparison to automated data (Figure 2). The reaction was initiated and aliquots were removed at various time points for a total of 60 min. The reaction proceeded to <20% in the course of both this PAGE experiment and the automated method (Figure 2). Analysis of 2 μM Pb^{2+} data by traditional PAGE methods provided a rate constant similar to that determined by the automated capillary sample introduction and chip separation method (see below).

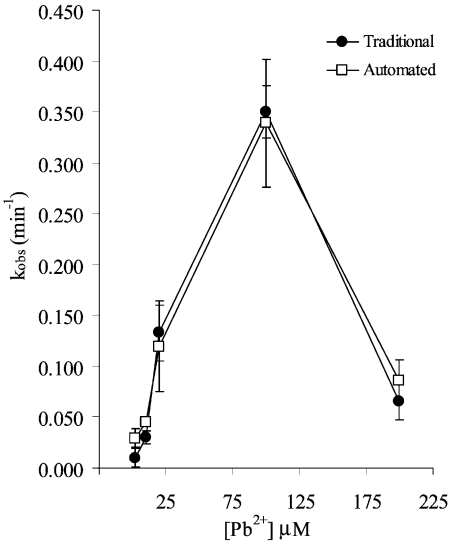

Figure 6. Observed kinetic rate constant comparison. Data collected using the automated gel electrophoresis system (□) compared to data collected using traditional PAGE methods (●). Leadzyme has a bell-shaped dependence on Pb^{2+} concentration. Each automated gel electrophoresis reaction was performed a minimum of three times for each Pb^{2+} concentration. The k_{obs} was determined as described in the Experimental Section for each of the five lanes, and data from 10 to 15 total lanes were averaged. The average ± the standard error of the mean (SEM) is reported for each Pb^{2+} concentration. Any data from individual lanes exhibiting baseline irregularity due to bubbles in the gel were excluded from the average. All kinetic information obtained by traditional PAGE is an average of two experiments ± SEM.

This PAGE method required a great deal of labor as it is not an on-line method. Sample collection, gel separation, and drying took ~2−3 h. Through the use of automated capillary sample introduction and microchip gel electrophoresis, the time required per experiment has been reduced to the length of time of the enzymatic reaction under investigation, 1 h for these experiments. This decrease in experiment time has been achieved by collecting and separating samples simultaneously. No further preparation is necessary once the separations are complete. Additionally, the automated separation system allows many experiments to be performed serially, thus eliminating the time required to set up each individual experiment, and making this a valuable high-throughput separation technique for RNA analysis. Overall, the automated separations system increased throughput by at least 2−3 times as compared to traditional gel electrophoresis.

Next we examined reaction kinetics under a range Pb^{2+} concentrations both to confirm the reported bell-shaped dependence of the leadzyme and to compare the results of the two methods. The automated experiments corroborate nicely with PAGE analysis (Figure 6) and exhibit the known bell-shaped dependence on Pb^{2+} concentration.[49] All k_{obs} values collected by the traditional and automated methods were in good agreement, differing by 2-fold or less. Importantly, by showing that comparable values are obtained with both techniques, PAGE analysis has validated the automated results.

CONCLUSIONS

Using a simple ribozyme model, the leadzyme, it has been shown that capillary sample introduction into a microfabricated chip is a promising technique for monitoring the cleavage, and therefore the reaction kinetics, of ribozymes. The ability to inject multiple times from a single reaction vial without sample cleanup or other sample-handling steps significantly reduces the time required for these experiments and improves reproducibility. Good sampling rates are achieved, and channel-to-channel reproducibility has been enhanced through the integration of an air-actuated valve for injection. Additionally, automated capillary sample introduction requires very little sample in terms of ribozyme and total reagent volume. The current limitation to this technique is the 53-s dead time between the reaction initiation and the time of the first data point. Kinetic rate constants were determined for the moderately slow leadzyme model system. These data show that the leadzyme is very sensitive to the concentration of Pb^{2+} present in the reaction mixture and correlate well with published trends.[49] Future work will involve the integration of a chip with an increased number of channels for a more thorough analysis of early time points. Additionally, the lag time between reaction initiation and capillary loading is under investigation to enable this technique for even faster kinetic reactions. This technique should be suitable not only for the analysis of the leadzyme but also for future analysis of more complex RNA and protein enzymes.

The ability to monitor complex ribozyme reactions in the laboratory is especially important since they represent more realistic models of what occurs in vivo. In addition, the ability to monitor multiple products is critical for future studies of more complex systems, and this technique provides a new way of continuously monitoring such dynamic processes. Continuous analysis of the products of a ribozyme cleavage reaction contributes to the fundamental understanding of ribozyme cleavage, stability, and structure, thereby enhancing practical knowledge for the use of ribozymes in the clinical setting. It is clear that the automated capillary sample introduction into a microfabricated chip separation technique presented in this paper is capable of monitoring ribozyme cleavage reactions and shows great promise for the analysis of more complex biological samples.

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