Design and Characterization of Microfluidic Analysis System for RNA-Aminoglycoside Interactions

Ji-Hye Lee¹,³, June Moon Jang², Han-Sang Cho², Ki-Cheol Han¹, Tae Song Kim², Ji Yoon Kang² and Eun Gyeong Yang¹,*

¹Life Sciences Division, Korea Institute of Science and Technology, Seoul, 136-791, Korea
²Microsystem Research Center, Korea Institute of Science and Technology, Seoul, 136-791, Korea
³Department of Life Science & Biotechnology, Korea University, Seoul, 136-701, Korea

*Corresponding author: eunyang@kist.re.kr

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Abstract. Microfluidic devices are of considerable interest, since such technology offers great promise for the development of powerful and versatile miniaturized analyzers. Accordingly, the present work describes a microfluidic screening system that is composed of a microchip, hydrodynamic pumping unit and fluorescence detectors. To develop an assay for RNA-aminoglycoside interactions, microchips are designed and fabricated on a glass substrate, then flow simulations are performed in the microchannels. After optimizing the flow control and buffer composition for fluorescence-based biochemical assays, a fluorescently labeled aminoglycoside probe and RNA are allowed to flow continuously to the main micro-channel based on hydrodynamic pumping and their interactions monitored by fluorescence quenching, which is reversed upon competition with other aminoglycosides. Consequently, the proposed device can serve as an integrated microfluidic platform for the high-throughput screening of high affinity antibiotics for RNA targets.

Introduction

The trend towards the miniaturization of chemical and biotechnological analysis systems and rise of the ‘lab-on-a-chip’ concept has generated a great deal of interest in the scientific community and led to the rapid expansion of the research field of microfluidic systems [1]. The applications of microfluidic systems are numerous, and it is increasingly clear that microfluidic systems are becoming an important tool for many chemical and biochemical studies [2]. As the screening of large compound libraries against drug target molecules requires high-throughput methods, microfluidic analytical devices have also been applied to drug screening [3]. These devices offer significant advantages over standard laboratory instrumentation, including design flexibility, dimensional precision, and reduced consumption of reagents and power. Accordingly, the present work demonstrates the feasibility of an on-chip assay based on RNA-aminoglycoside interactions integrated in a microfluidic system. RNA molecules can form intricate three-dimensional structures [4], and since these structured RNA molecules have dynamic biological functions and therapeutic potential, it is important to select high affinity antagonists directed against these structures for drug discovery [5]. As such, in the current study, a microfluidic analysis system was constructed, chips designed and simulated, and an on-chip assay developed using an RNA-binding fluorescently-labeled paromomycin probe. The resulting system was found to provide easy control and transport of fluids within a fluidic channel network, allowing integration and automation for an assay and exhibiting potential for drug screening.
Materials and Methods

Reagents. The 5-carboxytetramethylrhodamine labeled paromomycin (CRP; Fig. 1) was prepared from paromomycin sulfate and 5-carboxytetramethylrhodamine (TMR) succinimidyl ester, as previously reported [6]. To make a truncated 78-mer telomerase RNA (Fig. 1), a synthetic oligonucleotide DNA template was amplified by a PCR. The RNA was then in vitro transcribed by T7 RNA polymerase and purified by gel electrophoresis, followed by quantification spectroscopically at 260 nm. The reagents were all purchased from Sigma-Aldrich, except for polyvinylpyrrolidone (PVP) and dimethylethylammonium propane sulfonate (NDSB-195) that were from Calbiochem. All the buffers were filtered through 0.22-µm disposable Millipore syringe filters.

Fig. 1. Structure of CRP (left) and Mfold [7] minimized secondary structure of telomerase RNA (right)

Chips. Standard semiconductor photolithographic techniques were used to manufacture the glass chips [8, 9]. Briefly, poly-silicon was deposited on a Corning 7740 Pyrex glass substrate (Corning Co.) using low-pressure chemical vapor deposition as a mask against HF etching. AZ4620 Photoresist (Clariant Corp.) was then spin-coated, soft-baked, and selectively exposed to ultraviolet light. The resulting pattern was transferred into the poly-silicon layer by reactive ion etching using Plasma Therm 790 (Plasma Therm Inc.). The channels on the glass wafer were then wet-etched with a 49% HF solution, followed by the removal of the poly-silicon. Two-mm-diameter through-holes were etched-out with a sand blaster as reservoirs and a 380 µm hole created for capillary attachment on a second layer of glass. The two glass substrates were then thermally bonded to create a fluid network and reagent well arrays. After inserting and bonding a diced capillary to the hole, the chip was thoroughly cleaned with deionized water and 2-propanol.

Instrumentation. A biochip probing & detection system was custom-made, as depicted in Fig. 2. A specially constructed holder for the chips was then placed on the stage of a fluorescent microscope, a mercury arc lamp used as the light source, and the excitation and emission wavelengths selected based on an appropriate filter set. The fluorescence images were collected using a digital CCD camera (Hamamatsu Photonics KK) and analyzed with image software on a PC. The fluorescence data was also collected using a photomultiplier tube module (Hamamatsu Photonics KK) equipped with a vacuum pump for hydrodynamic control and handler (Robots and Design Co. Ltd.) for positioning the well plates.

Simulations and calculations. The designed chip was verified for bulk fluidic behavior using simulation software Coventorware (Coventor Inc.), while the mixing of the inlet flows was analyzed using an analytical solution of diffusion equations [10].
On-chip experiments. Before use, the chips were cleaned with 1 N NaOH, 0.1 N HCl, deionized water, and a run buffer for 10 min. The chips were then filled with the required buffers, placed in the holder, and mounted on the microscope stage. Next, the microscope was focused on the detection region of the chip. To sip the solution from the well plate, the capillary was aligned with the center of the well. Prior to the experiment, a high vacuum was applied to the chip to remove any bubbles. The data was then analyzed using the plotting program Origin.

Fig. 3. Layout (above) and geometric configuration (below) of microfluidic chip

Results & Discussions

Fluorescence-based microfluidic chip detection system for screening. Fig. 2 shows a schematic of the proposed microfluidic chip detection configuration that can be used for fluorescence measurements. The system consists of a pump, detector unit, and chip and plate handling stages. The detector includes a light source for excitation, light focusing, and fluorescence collection optics, a microscope, and photomultiplier tube. To calibrate the detector, the intensity was measured in fluorescence units as a function of the TMR, which showed a linear relationship with a lower detection limit of ~100 nM (data not shown). The delivery of the samples from the well plates through the capillary to the micro-channel was automated using a plate handler, while a 3-axis robot with a transport precision of 0.001 mm controlled the position of the plate under the chip. To drive the fluids through the micro-channel hydrodynamically, the pressure controller was optimized to apply a vacuum to the waste reservoir of up to –69 kPa with a resolution of 0.34 kPa.

Chip & mixing simulations. A schematic diagram of the chip design including the geometric configuration, is shown in Fig.3. The chip was designed so that the CRP probe mixes downstream into the buffer containing the chemical supplied from the well plates through the capillary, while the RNA mixes into the stream later. As such, any reactions or binding events were expected to occur in the main channel. For the chip design, the ratio of the three inlet flows (capillary:CRP:RNA) was set at 70:15:15, while the reaction time from the junction to the detection point was anticipated to be 25 sec. The designed chip was fabricated and its dimensions confirmed by SEM; the depth of the channel was ~12 µm and the widths of the inlet and main flow were ~29 µm and ~74 µm, respectively. The chip was also verified using the same simulation software used to calculate the bulk fluidic resistance.
model. The time taken to deliver the sample plug from the capillary tip to the detection point was calculated and plotted as a function of pressure, as shown in Fig. 4. For a pressure drop of ~13 kPa (2 psi) from the waste well to the capillary, the chip provided a delivery time of ~25 sec, which was consistent with the measured delivery time (Fig. 4). Next, the on-chip mixing of the inlet flows perpendicular to the flow direction was analyzed. Since the fluid flow in microfluidic channels is typically characterized as laminar, fluid streams parallel to each other will only mix through convective and molecular diffusion. Therefore, the analytical solutions of diffusion equations were employed to perform mixing simulations [10]. The diffusion coefficient for RNA was approximated to be 70 \( \mu m^2/sec \), while those for CRP and chemical from the capillary approximated to be 100 \( \mu m^2/sec \) [11]. The concentration profile for telomerase RNA with respect to time (Fig. 5) showed that the RNA flow diffused completely across the channel in about 25 sec, while the CRP and chemical appeared to fill up the channel in 21 sec and 9 sec, respectively (Fig. 5). When red and blue dyes from the reagent wells of the chip were allowed to flow into the main channel, complete mixing of the two dyes across the channel was observed less than 10 mm from the point of the RNA inlet to the main stream. Accordingly, these data indicate that the dimensions and structure of the chip along with the pressure-driven flow are compatible with the study of RNA-aminoglycoside interactions.

**Sticking issues and buffer optimization.** The ratio of surface area to volume for microfluidic devices is higher than for any other assay format, such as microtiter plates. In addition, the surface of the chips contains a high density of silanol groups, which can cause sticking problems for certain reagents used in assays on microchips. For the probe molecule in the current study, the CRP appeared to interact with the surface, as shown by peak-broadening and long-tailing, whereas the TMR did not seem to interact with the glass surface (data not shown). This was probably due to the highly positively charged aminoglycoside portion of the CRP probe, resulting in electrostatic interactions with the negatively charged chip surface. Attempts were made to reduce or eliminate these interactions by adding various components to the assay buffer, including buffering reagents, high salt...
concentrations, detergents, and surface coating reagents (data not shown). Certain additives, such as detergents, including Brij-35 and Triton X-100, BSA, and PEG did not show any effect on the problems associated with CRP adsorption. Meanwhile, when HEPES was replaced with phosphate as the buffering agent, the problems were partially alleviated. In addition, the shape of the peak became narrower when the channel was dynamically coated with 0.3% PVP, suggesting that PVP coating may have neutralized the negatively charged surface of the channel, thereby reducing the interaction between the CRP and the channel surface. Furthermore, the addition of a non-detergent zwitterionic compound NDSB also reduced the problems. Therefore, a buffer containing 20 mM NaPi, 140 mM NaCl, 1 mM MgCl$_2$ and 5 mM KCl, 1M NDSB, pH 7.4 was used for the chip assays.

**On-chip assay.** To generate stem-loop secondary structures of RNA, which are known to be important for binding with aminoglycosides [12], the telomerase RNA was heated to 65 °C for 5 min, then cooled slowly to room temperature. At this point, the RNA molecule was expected to exist in the secondary structure shown in Fig. 1. Initially, CRP was added to the well of the chip, continuously driven to the main channel by hydrodynamic flow, and the fluorescence observed (Fig. 6). Upon addition of the pre-formed RNA to the well of the chip, the fluorescence signal was significantly quenched (Fig. 6). This observation is consistent with previously reported data measured by conventional fluorescence measurement assays using a fluorescence spectrometer [12]. When neomycin was briefly injected from the plate well through the capillary to the fluid in the main channel, almost 75% of the original fluorescence intensity of the fluid containing CRP only was recovered, and the quenched level restored (Fig. 6). Therefore, these results indicate that the neomycin bound to the telomerase RNA as a competitor and released the free CRP into the fluid, thereby leading to the observed recovery of fluorescence intensity. These effects were also confirmed using a fluorescence spectrometer (data not shown).

![Fig. 6. Fluorescence intensity changes observed (A) by microscopy and (B) PMT upon binding of CRP (10 µM) with telomerase RNA (25 µM) at -13 kPa and competition with neomycin (10 mM) sipped for 10 sec](image)

**Summary**

This paper presented a new biochip strategy for studying RNA-aminoglycoside interactions using a single-channel microfluidic device. In addition to setting up the instrument and chip systems, the flow control is analyzed and an on-chip competition assay for RNA-aminoglycoside interactions demonstrated using a hydrodynamic flow. Future studies will use the developed on-chip assay system for the high-throughput screening of antibiotics for RNA targets. The proposed strategy can also be employed to design microfluidic chips with appropriate structures and dimensions for other biochemical experiments.
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References


