SMALL, COST-EFFICIENT STOPPED-FLOW DEVICE

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ABSTRACT

Conventional Stopped-Flow devices often need high sample volumes, display complex designs, take up much space and are very expensive in purchasing and maintenance. Here, a powerful microfluidic based Stopped-Flow system is described that is flexible in application, simple, easy to handle, and cost-efficient in terms of acquisition and operation. With a dead time of just 7 ms, the ability of kinetic measurements in absorption as well as fluorescence mode over a wide wavelength range, and the monitoring of intrinsic protein fluorescence without additional dyes at 280 nm, this small system is competitive to commercial available systems.

KEYWORDS: Stopped-Flow, microfluidics, cost-efficient instrumentation, low dead time

INTRODUCTION

Already developed in 1940 [1] Stopped-Flow is an established technology for high time-resolved monitoring of rapid (bio)chemical reactions like dynamic investigations of enzyme reactions, protein interactions or molecular transport mechanisms. Conventional Stopped-Flow devices often display a complex design, take up much space and are very expensive in purchasing and maintenance. The need of high sample volumes of often expensive substances for each experiment is another disadvantage of these systems. Here, a powerful microfluidic based Stopped-Flow system is described that combines the advantages of a simple, low-cost set-up with low sample consumption based on a disposable polymer chip. The realisation of microfluidic disposables not only helps to minimise sample volumes but also guarantees easy handling and elimination of interferences by cross-contamination. The system contains a high-precision valve system for fluid control as well as an automated high time-resolved data acquisition. Applying standard reactions the reaction kinetics are followed by transmission or fluorescence measurements and thereby the dead time of the system was determined to be just 7 ms. In combination with the ability of kinetic measurements in absorption as well as fluorescence mode over a wide wavelength range, and the possibility to monitor intrinsic protein fluorescence without additional dyes at 280 nm makes this system competitive to commercial available systems.

THEORY

Hence, Stopped-Flow is a well established technology which is widely used for the investigation of fast (bio)chemical reactions. An efficient and fast mixing process coupled with a sensitive optical detection system which allows for data acquisition with high time resolution is needed to perform such time resolved measurements. In commercial Stopped-Flow devices the demands of high performance measurements end up in complex and costly designs. Individual components like light source, amplifier electronics, detection unit and mixing station take up much space and are very expensive in purchasing and maintenance. Based on the numerous mechanical components these systems are susceptible to faults. Conventional setups usually harbour two step motor-driven syringes to transport the reactants (e.g. enzyme and substrate) with very high speed over a mixing chamber directly into the optical detection vessel. The detection unit is triggered by a stop syringe. Monitoring changes in absorption or fluorescence intensity the processes within the vessel can be analysed. The time between the point where reactants meet first and the start of the data acquisition of the reaction is called dead time. Only processes slower than the dead time can be monitored. The dead time is mainly dependent on the fluid flow rate, the mixing efficiency, the volume between mixing chamber and detection vessel and the volume of the detection vessel [2].

Often, relatively high sample and reagent volumes at least for the first operation of the system are needed (up to 3 ml for initial filling and up to 1 ml for each experiment). Since the samples and/or reagents are often expensive or isolated under extreme effort this first volume needed is an intrinsic problem of conventional devices. Nevertheless, in a series of experiments that work without change of chemicals these systems can be very volume efficient with respect to each single experiment. Usually, extensive test series with various parameters are performed, and therefore considerable amounts of reactants are needed. Thereby, flushing and washing steps are the most volume consuming steps. Motivated by this a microfluidic based Stopped-Flow system has been developed combining the advantages of a simple and cost effective set-up with low sample volume requirements. The realisation of microfluidic disposables not only helps to minimise sample volumes and costs but also guarantees easy handling and elimination of interferences by cross-contamination.

EXPERIMENTAL

Fig. 1 shows a schematic diagram of the microchip-based stopped flow device. Solutions A and B are pushed into the chip using syringe pumps up to stop valve V6. The whole system is thereby filled with liquid free of air bubbles. After switching valves V4 and V5, V1 is opened for a predetermined time (e.g. 20 ms) to drive both solutions into the cuvette by air pressure (2 bar). The flow is actively stopped by simultaneous closing of valves V1 and V6. Data acquisition starts with pumping to monitor the complete process. All measured data are stored for subsequent analysis. After the measurement V6 is opened, followed by V2 to vent the system. An additional valve and pump was integrated into the system

ment V6 is opened, followed by V2 to vent the system. An additional valve and pump was integrated into the system to allow washing the system after each experiment. To pump a wash buffer through the system valve V3 has to be opened.

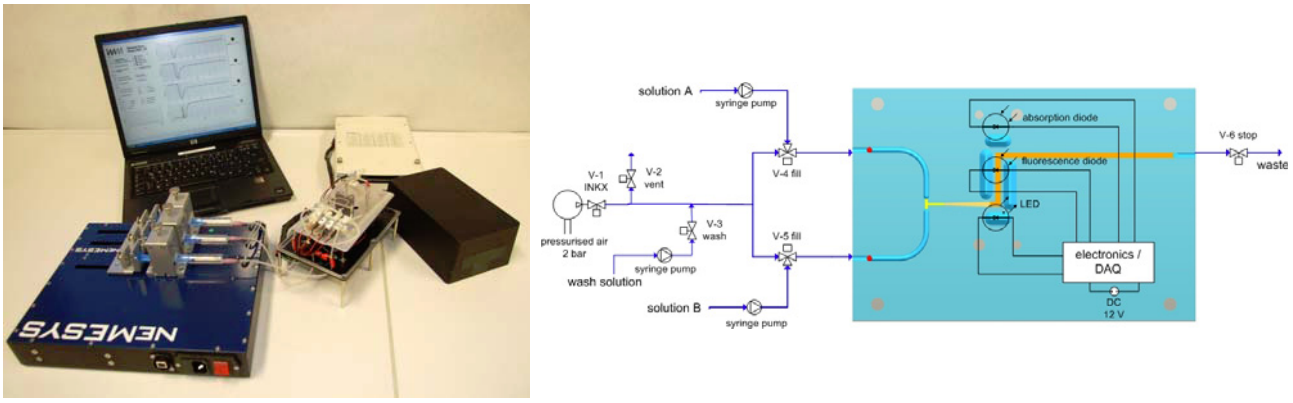


Figure 1: Realised Stopped-Flow set-up and schematic diagram of the microchip-based stopped-flow device.

For the instrument electronic controlled high-precision magnetic valves especially developed for miniaturised instruments were used. They combine the advantages of very fast response time (<1 ms) and low dead volume (few µl) with low power requirement (12 V). In addition, valves resistant against pressures up to 120 psi (ca. 8 bar) are available. Since these valves are error-prone by small particles filtration of the liquids used is recommended (10 µm pores). Sample loading and pulsation-free pumping is realised by using syringe pumps.

The detection unit consists of a light emitting diode for excitation, photodiodes for absorption and fluorescence detection. The photodiodes are equipped with a cut off filter or band-pass interference filter for wavelength selection. Since the photoelectric current of the diodes is in the pico- to microampere range a multiboard was used for signal enhancement and transfer of current to voltage (Enhancement Volt-to-Volt or Ampere-to-Volt, amplification factor 10^7 V/A (independent) or 10^{10} V/A (in-line), limited output signal of 0-4 V, dual power requirements of ±9 V…±24 V). Due to the high signal amplification needed the whole system had to be shielded. The aluminium cover also serves as darkening for the fluorescence measurements.

For the interconnection between device and computer a multi function data acquisition board was included. Main features are the analogous input ports for data acquisition from the optical sensor and the digital output ports for the precise valve control in the millisecond range. Data are collected with selectable sample rate and measurement time (limit: 80 ksamples/sec). For instrument control software *LabVIEW* Version 8.6 was used. On the user interface diverse parameters can be set individually. For example, response times of valves can be adapted to guarantee fresh reactant within the cuvette at the onset of measurement. In addition, sampling intervals as well as number of readings and therefore total measurement time can be set according to the kinetics of the investigated reaction.

RESULTS AND DISCUSSION

Performance and dead time of the developed microchip-based Stopped-flow device were determined by applying three different reactions: 1) reduction of 2,6-Dichlorophenolidophenol (DCIP) by ascorbic acid via absorption (Fig. 3, [3]), 2) fluorescence quenching of N-acetyltryptophanamide (NATA) by N-bromosuccinimide (NBS) (Fig. 4, [4]) and 3) labeling of HDAH (Histone deacetylase) with fluorescent dye Fluorescamine (Fig. 5, [5]). Also, measurements in the UV range are possible with the system, e.g exitation at 280 nm for investigations of the intrinsic fluorescence of proteins. Determination of time constants for reactions 1) and 2) showed similar data as published in literature. The resulting dead time is about 7 ms and competitive with conventional Stopped-Flow systems. For reaction 3) published kinetic parameters were reproduced consistent with the published reaction scheme [5].

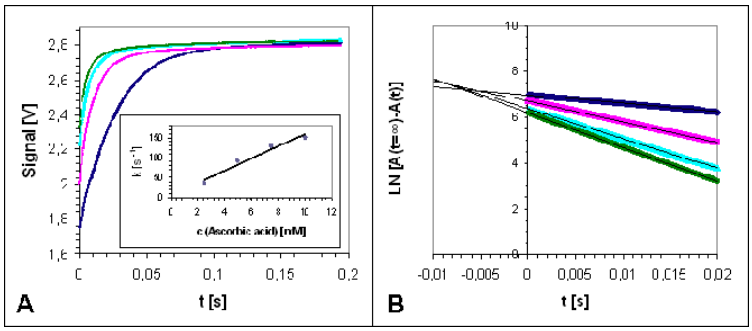
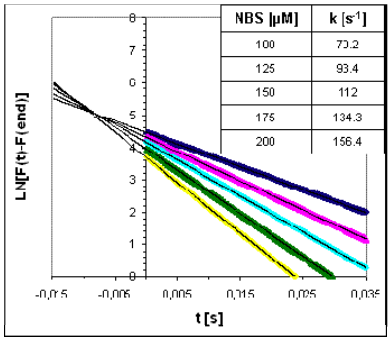


Figure 3: Reduction of 2,6-Dichlorophenolindophenol (DICP) by ascorbic acid. (A) Average of seven independent measurements per ascorbic acid concentration (70 µM DCIP + 2.5 mM (blue), 5 mM (pink), 7.5 mM (turquoise), 10 mM (green)), relationship of rate constant and ascorbic acid concentration R^2=0.9611, (B) dead time plot of linearised curves in (A); resulting dead time is ca. 7 ms.



THE EFFECT OF THE NBS CONCENTRATION ON THE FIRST-ORDER RATE COEFFICIENT (k) FOR THE QUENCHING OF N-AcTrpNH$_2$ FLUORESCENCE[a]

[NBS]/M	k/s^{-1}	[NBS]/[NBS]$_0$	k/k_0
5×10^{-5}	37.4	1	1
10×10^{-5}	70.5	2	1.88
20×10^{-5}	155	4	4.14
30×10^{-5}	216	6	5.78

[a] $[NBS]_0$ is the NBS concentration of 5×10^{-5} M and k_0 is the first-order rate coefficient of 37.4 s^{-1}. Conditions of the experiment are the same as described in the caption of Fig. 1.

Peterman (1979). Anal. Biochem. 93; 442-444

Figure 4: Quenching of NATA (5 µM) by NBS, pH 7, 23°C: Average of four independent measurements per NBS concentration (100 µM (blue), 125 µM (pink), 150 µM (turquoise), 175 µM (green) 200 µM (yellow)); Determined first-order rate coefficient k for the quenching of NATA fluorescence by NBS with the developed Stopped-Flow system is in accordance with published data.

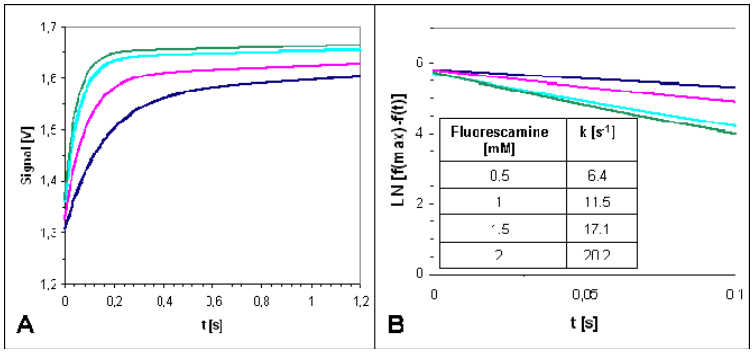


Figure 5: Labeling of HDAH with Fluorescamine: 3 µM HDAH mixed 1:1 with Fluorescamine in DMSO (4 mM (green), 3 mM (turquoise), 2 mM (pink), 1 mM (blue)), 22 °C. (A) Average of four independent measurements for each Fluorescamine concentration. (B) Natural logarithm of the difference of maximal observed fluorescence intensity minus the intensity at a fixed time against time.

CONCLUSION

A small automised Stopped–Flow device is presented. Based on a microfluidic polymerchip and a compact custom-made instrument the system is flexible in application, easy to handle and cost-efficient in terms of acquisition and operation. Building up the instrument with conventional components in combination with an efficient, fast reacting multi function data acquisition board and a flexible user interface for high-precision valve control and automatic, continuous data acquisition lead to a small, compact and cost-efficient system. Due to the compactness of the system only small volumes of reactants – about 20 µl per measurement - are needed. Showing linear absorption measurements up to about $A_{490} = 1.5$ and a lower detection limit of Fluoresceine ($\lambda_{Ex} = 490$, $\lambda_{Em} = 530$ nm, amplification 10^7 V/A) at 50 nM the system is as efficient as conventional photometers. Work is ongoing to bring the detection limit of the system further down. Dead time was shown to be 7 ms and therefore again competitive with conventional systems.

An important detail for the robust fluid control within the system is the integration of stop valve V6 which guarantees defined active halt of the liquid within the detection vessel. This concept also avoids air bubbles within the cuvette even at applied pressure of up to 2 bar. The possibility to wash the system after each experiment allows for consecutive series of measurements within one single chip which lead to reliable data.

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