Lab-on-valve: universal microflow analyzer based on sequential and bead injection

Jaromir Ruzicka

Department of Chemistry, University of Washington, Seattle, WA 98115, USA

This paper introduces a novel methodology for downscaling reagent based assays to micro- and submicroliter level. It is shown that sample handling in the sequential injection mode, which employs forward, reversed and stopped flow, can be programmed to accommodate a wide variety of assays within the same microfluidic device. Solution metering, mixing, dilution, incubation and monitoring can be executed in any desired sequence in a system of channels, integrated with a multipurpose flow cell. The channel system and flow cell are fabricated as a monolithic structure mounted atop a conventional multiposition valve. In addition to compactness, the advantage of this 'lab-on-valve' system is the permanent rigid position of the sample processing channels that ensures repeatability of microfluidic manipulations, controlled by conventional sized peripherals. With the exception of the integrated microconduit system, that has been designed and mesofabricated by computer aided design (CAD) technology, all peripherals (sequential injection system, fiber optic UV/VIS spectrophotometer-fluorometer) are conventional sized and commercially available components. This provides proven robustness and reliability of operation, and makes the microfluidic system compatible with real life samples and peripheral instruments. The system has been characterized by dye injection, to provide guidelines for method development. Its versatility is documented by a phosphate assay, enzymatic activity assay of protease and by a bioligand interaction assay of immunoglobulin G (IgG) based on its interaction with protein G immobilized on Sepharose beads.

Introduction

The promise of revolutionizing reagent based (bio)chemical assays through the automation of the microfluidic handling of samples within small integrated units has inspired a rush of scientific[1,2] and commercial activities aimed at the 'lab-on-chip' development.[3] Taking the cue from the tremendous impact of microminiaturization on electronics, a similar path is being advocated for analytical fluidics, where the downscaling of the processed volumes from mL to fL in channels ranging from 1000 to 1 μm is being pursued.[1–4] Since the current focus is mainly on devices in the nL to pL range, often accompanied by a desire to miniaturize and integrate valves, pumps and reagent reservoirs on the same chip, novel techniques for fluid pumping (electrokinetic, pneumatic or by centrifugal force), mixing and detection have been proposed,[1–4] yet their practical implementation remains a challenge. Ironically, current trends in microassays are increasingly being focused on analyzing macromolecules [DNA, immunoglobulin G (IgG), proteins] in samples containing suspended matter (colloids, cells, particles) which is increasingly problematic as the microfabricated channels become narrower and longer.

Keeping in mind that the ultimate goal of microminiaturization of reagent based assays is the decreased use of materials consumed and waste generated, only the volume of the *sample processing channel* needs to be minimized. Moreover, the ultimate limiting factor to miniaturization is not our ability to fabricate minuscule conduits, but the smallest detectable quantity of the target analyte. For spectroscopic measurements, this translates into the smallest acceptable detector volume, as pointed out a long time ago in a classical study by van der Linden.[5] Indeed, the most widely used spectroscopies require a minimum light path length of 1 mm in order to achieve a reasonable sensitivity, which in turn necessitates sample volumes in the μL or upper nL range. Since the goal of this work is to introduce a universal system applicable to a wide range of reagent based assays using spectroscopic techniques, the present version of the 'lab-on-valve' system has been designed to operate at the μL level with a downscaling option to the nL range, while maintaining relatively large bore conduits that minimize surface contamination and clogging. The additional benefit of this choice is robustness, offered by conventional sized pumps, valves and fiber optic spectrometers.

It follows from the foregoing discussion that the central sample processing unit (CSPU) must be designed in such a way that the sample path from injector to flow cell must be *minimized, yet easily variable*, in order to accommodate sample metering, dilution, reagent addition, mixing, incubation, separation and detection, ideally in *any* desired sequence. While this is not possible to achieve with a continuous unidirectional flow (a current mode of operation of all μTAS (micrototal analysis systems) flow based devices,[1,2] it is quite feasible in a sequential injection (SI) mode that employs flow programming based on reverse, stopped and forward flow.[6,7] Samples and reagents are injected by means of a multiposition valve (Fig. 1) by flow reversal into a holding coil, where mixing dilution and incubation take place. During measurement, a forward flow is applied to transport reactants into the flow cell, where the flow is then stopped to monitor the reaction rate. Use of a multiposition valve allows random access to a sampling port (#5) and to reagent reservoirs (#3, #4, #6). Miniaturization is achieved by designing a CSPU that integrates the sample processing channels with a multipurpose flow cell (Fig. 2).[8] The salient features of this design are: (i) close proximity of the injection port (#2) to the flow cell; (ii) multipurpose flow cell (Fig. 3); (iii) flexibility of sample processing, achieved by reversing the flow into the holding coil and by random access provided by the multiposition valve; and (iv) an improved configuration of the jet ring flow cell (Fig. 3, D–F).

Experimental

Instrumentation

Apparatus. Microfluidic manipulations were controlled by an FIAlab 3000 system [FIAlab Instruments Inc. (formerly Alitea USA), www.flowinjection.com], equipped with an Ocean Optics S 2000 spectrophotometer-fluorometer (www. oceanoptics.com). The system was controlled using a Dell Dimension XPS T450 PC, operating FIAlab software (5.3 version). The deuterium and tungsten light sources (Ocean Optics) as well as the spectrophotometer-fluorometer were connected with the flow cell by standard FIAlab fiber optic cables. The illuminating fiber and the collecting fiber for fluorescence and UV measurement has od of 150 µm, and the
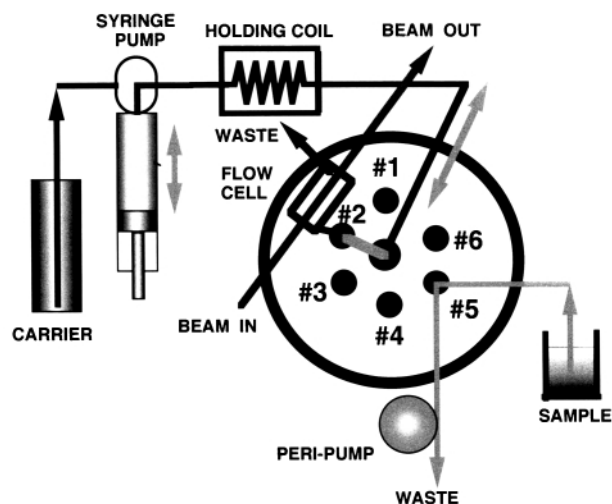

Fig. 1 Micro SI system comprising a high precision syringe pump (500 µL volume) and an auxiliary peristaltic pump, shown serving the flow through sampling port (#5). The central sample processing unit, integrated with a flow cell (FC), is mounted atop a six-position valve. The syringe pump operates in reversed–stop–forward flow sequences, for metering the sample (#5) and the reagents (#3, 4, 5) into the holding coil. Flow reversal is used to direct the reaction mixture into the flow cell through port #2. The flow through cell is shown in absorbance configuration (Fig. 3A,B).
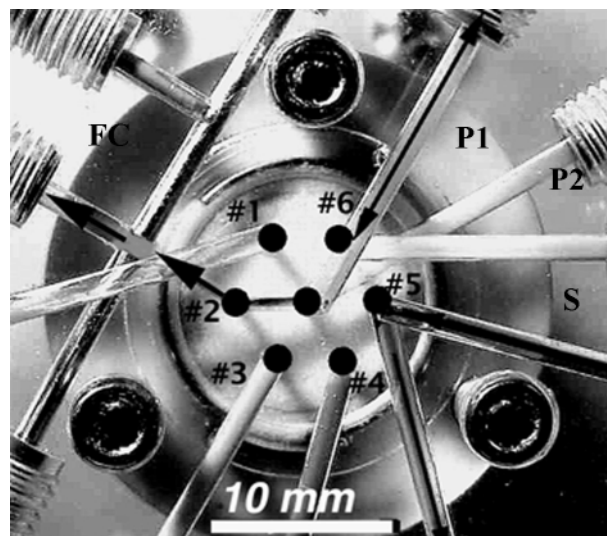

Fig. 2 CSPU ('Lab-on-valve') shown mounted atop a six-position valve. P1, P2 are channels leading to holding coils and syringe pumps. Sample S is shown in a flow through sampling port (#5) that is connected to the sample container and peristaltic pump. The flow cell (FC) is shown in absorbance configuration using two optical fibers facing each other (1.5 mm light path). Arrows leading from P1 through #2 and into the flow cell indicate the valve position during the transport of analyte into the flow cell.
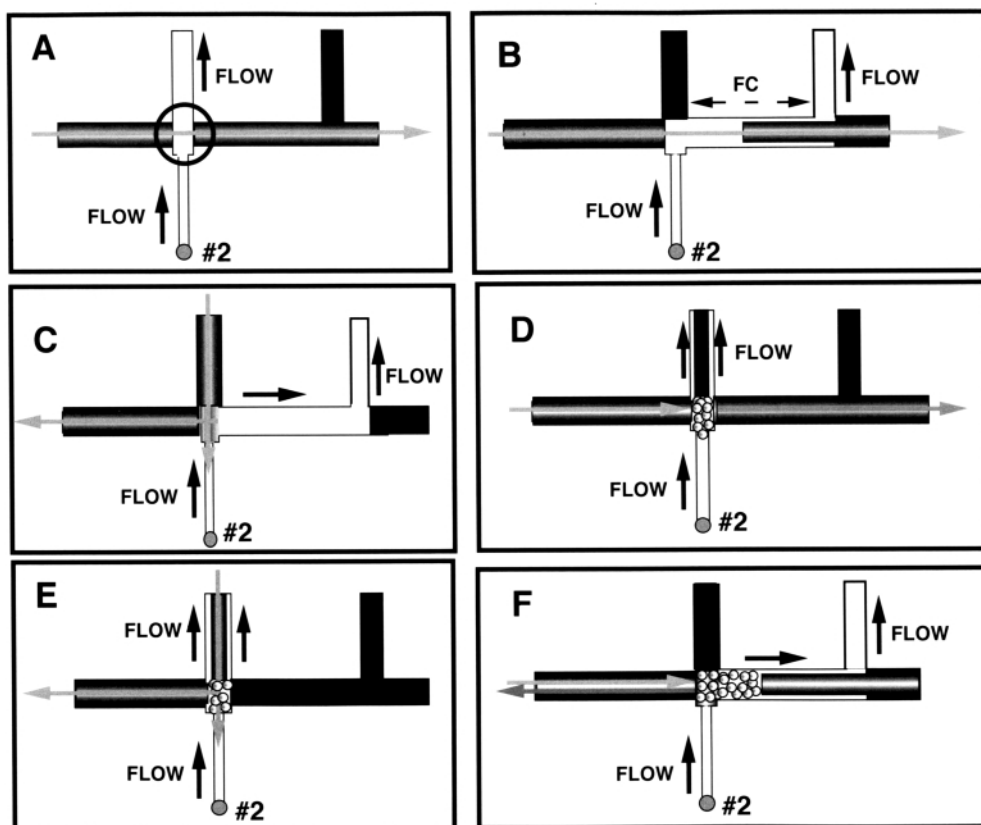

Fig. 3 Multipurpose flow cell, integrated to port #2, uses optical fibers encased in stainless steel tubing, that is proportioned to leave a 30 µm gap between the casing and channel walls. Since the channels are terminated at the perimeter of the CSPU by fittings (Fig. 2), the fibers can be readily reconfigured for absorbance (A, B) and fluorescence (C) measurement. Since the 30 µm gap allows liquid to escape, but retains beads, the flow cell can also be assembled to a jet ring configuration for absorbance (D), fluorescence (E) and reflectance (F) measurement. Black blocks indicate filled and closed channels.

collecting fiber for VIS spectrophotometry uses a 50 µm od fiber.

CSPU. The CSPU shown in Fig. 2, the final result of numerous computer aided design (CAD) prototypes, was mesofabricated from a single block of Perspex, 15 mm thick and 60 mm od. The channels inside the block are situated in three planes. Consequently, the channel connecting port #1 is above the channel connecting port #2 to the flow cell. Similarly, the channel leading to port #6 is situated in a layer in between the two channels P1, P2, leading to the central port. (Channel P2, designed for the second syringe pump,[9] is not used in this work.) The channel connecting the central port with the flow cell, marked by a short arrow, has an id of 500 µm and an approximate volume of 6 µL. All other channels have 1/16 in id (1.57 mm) and are terminated at the CSPU circumference by standard 10-32 thread to allow the use of standard tubing and fittings (Upchurch Scientific, www.upchurch.com). In this work, 500 µm id PEEK or Teflon tubing was used for all connections, being inserted all the way into the CSPU as close to the ports as possible. This allows for the downscaling of the system, since tubing is available in several smaller id values down to 75 µm. Thus the id of the CSPU channels is variable or can be filled up and closed (such as P2) by substituting a rod instead of the tubing. The channel may also be left fully open, when the tubing is not fully inserted (see port #1), if extensive sample dilution is planned. Such a wide bore channel can also be used to accommodate a microcolumn, as outlined in the Conclusion.

Flow through port. This is made by connecting the two channels at port #5, one channel serving as the sample solution inlet, while the other channel serves as the sample outlet. An auxiliary peristaltic pump (Fig. 1) allows the sampling conduit to be thoroughly washed between samples of different concentrations, thus preventing carryover and assisting in increasing the sampling frequency. Sample consumption is also minimized, since air segmentation can be used when aspirating the sample solution from the sample container through the flow through port. Note that the sample injection is carried out by precision reversal of the syringe pump, immediately after the

pivoting groove of the multiposition valve has been moved to port #5.

Reagent reservoirs. These were made of 1 or 2 mL disposable plastic syringes, furnished with 500 µm id tubing, mounted in the peripheral fittings of the CSPU. This allows convenient exchange of reagents as required by different assays.

Multipurpose flow cell. This is permanently connected to port #2 as shown in Fig. 2. The illuminating and collecting optical fibers are encased in 1/16 in od stainless steel casings and secured in position by standard ferrules and fittings. This allows the flow cell to be configured for absorbance (Fig. 3, A,B) and fluorescence (Fig. 3C) measurement. The tolerances between the stainless steel casing and the 1/16 in id channel have been selected to leave a 30 µm circular gap, that allows liquid to escape, retaining beads larger than 30 µm in the flow cell. This feature allows the flow cell to be configured as a jet ring cell,[7,10] where the change in bead optical properties can be monitored by absorbance (Fig. 3D), fluorescence (Fig. 3E) or reflectance (Fig. 3F) spectroscopy. Due to the small volume of the channel between the flow cell and port #2, the beads can be effectively discarded by a short burst of flow reversal into the holding coil and then to waste (port #1). This avoids the necessity of moving the optical fiber electromagnetically in order to open the jet ring gap for bead ejection by forward flow, an approach used previously.[11] All experiments were carried out at room temperature (22 °C), yet it would be desirable to bring the holding coil and CSPU to a constant and elevated temperature.

FIAlab software. This was used without modification. It integrates flow programming with spectrometer control, data collection and evaluation. It allows for the editing of response curves, including the overlay of different experimental runs. Examples of software protocol are shown in Table 1, while the configuration of the apparatus and videoclips of the CSPU during an experimental run are available at www.flowinjection.com.

Table 1 Two reagent stopped flow assay. Experimental and software protocol[a]

Loop Start (#)12	'REAGENT #1	'STOPPED FLOW
Chemical New Sample	Valve molybdate	SyringePumpA Stop
Chemical Name Phosphate	SyringePumpA Flowrate (µL/sec)10	Delay (sec) 40
'Chemical Quantity 0to40	SyringePumpA Aspirate (µL) 10	'SAMPLE PORT WASHOUT
'STARTUP	SyringePumpA Delay Until Done	Valve sample
Spectrometer Reference Scan	'REAGENT #2	SyringePUmpA Aspirate (µL) 5
SyringePumpA Flowrate (µL/sec) 100	Valve ascorbic	SyringePUmpA Delay Until Done
SyringePumpA Valve In	SyringePumpA Aspirate (µL) 15	'SYSTEM WASHOUT
SyringePumpA Fill	SyringePumpA Delay Until Done	Valve flow cell
SyringePumpA Delay Until Done	'MEASUREMENT	SyringePumpA Flowrate (µL/sec) 50
'PREWASH	Spectrometer Reference Scan	SyringePUmpA Dispense (µL) 150
Valve flow cell	Valve flow cell	SyringePumpA Delay Until Done
SyringePumpA Valve Out	Spectrometer Reference Scan	Spectrometer Stop Scanning
SyringePumpA Dispense (µL)150	SyringePumpA Flowrate (µL/sec)5	'END
SyringePumpA Delay Until Done	SyringePumpA Dispense (µL) 30	Beep
Spectrometer Reference Scan	SyringePumpA Delay Until Done	Loop End
Spectrometer Absorbance Scanning		
'SAMPLE INJECTION		
Valve sample		
SyringePumpA Flowrate (µL/sec) 5		
SyringePumpA Aspirate (µL) 4		
SyringePumpA Delay Until Done		
Spectrometer Reference Scan		

[a] FIAlab software uses the above text as written above to control all operations. For phosphate assay, the value positions have been assigned: #1 waste, #2 flow cell, #3 blocked, #4 ascorbic acid, #5 sample and #6 molybdate. Sample solution is aspirated into throughflow port #5 by the auxiliary peristaltic pump at the beginning of the first loop. For duplicate injection, the next sample can be aspirated during each second cycle at any time between 'REAGENT #1' and 'SAMPLE PORT WASHOUT'. If the injected sample volume is 10 µL or larger, the step 'SAMPLE PORT WASHOUT' can be avoided.

Reagents

Dye injection. A 1 ppm solution of fluorescein was prepared in TRIS-HCl buffer (0.10 M, pH 8.5). The same buffer was used as the carrier solution. A bromothymol blue 200 ppm solution was prepared in a borax buffer (pH 9.2) (0.005 M $Na_2B_4O_7$). The same buffer was used as the carrier solution.

Phosphate assay. An ammonium molybdate 6.9 g L^{-1} solution in nanopure water was prepared. An ascorbic acid 8% solution in nanopure water was prepared freshly each day and mixed with an equal volume of 0.05% solution of potassium antimony(III) tartarate ($KSbOC_4H_4O_6.1/2H_2O$). The carrier solution was citrate buffer (pH 3) (0.04 g Na_3 citrate.$2H_2O$ + 0.18 g citric acid.$1H_2O$ in 100 mL H_2O). This carrier solution also contained 0.01% of P20 surfactant (Biosensor, Uppsala, Sweden, catalog #BR-1000-54). Orthophosphate standards were prepared by dissolving 0.220 g of anhydrous KH_2PO_4 in 50 mL of nanopure water and by serial dilution of this stock to ppm P level.

Enzymatic assay. The enzyme savinase (EC3.4.21.14) was obtained as 1% pure enzyme powder, with a certified activity of 3.17 KNPU g^{-1} (KNPU, Kilo Novo Protease Unit[12]). It was dissolved in TRIS buffer to a stock solution of activity of 0.010 KNPU mL^{-1}. Serial dilution standards in the range 0–10 mK PU mL^{-1} were prepared. A working solution of fluorigenic substrate, AAF (7-[N-α-succinyl-L-alanyl-L-alanyl-L-(phenyl-anyl) amino]-4-methylcoumarin) (4 mM) was prepared by dissolving the substrate in N,N-dimethylformamide; TRIS buffer was then added to a final concentration of 10% N,N-dimethylformamide. The buffer used for the substrate and enzyme dilution, as well as the carrier solution, was TRIS-HCl (0.10 M, pH 8.3) containing 0.010 M $CaCl_2$. All solutions were stored refrigerated and the substrate solution was protected from light. Enzyme and substrate were kindly donated by Novo-Nordisk, Copenhagen, Denmark.

Bioligand interaction assays. The carrier solution was HBS buffer (0.01 M HEPES (N'-(2-hydroxyethyl)piperozine-N-ethanesulfonic acid), 0.15 M NaCl, 3 mM EDTA and 0.005% v/v surfactant P20; BIA certified, Biosensor, Uppsala, Sweden). This solution was also used to dilute the anti-mouse IgG-FITC (FITC, fluorescein isothiocyanate) and to wash Sepharose beads prior to use. Anti-mouse IgG (Fab specific) conjugated with FITC (product #F5262 obtained from Sigma, www.sigma-aldrich.com) had an F/P molar ratio of 5.5 and protein concentration of 4.0 mg mL^{-1}. Sepharose 4B beads, with recombinant protein G covalently bound to their surface, were obtained suspended in PBS (10 mM phosphate buffered saline, pH 7.4) from Zymed Laboratories (www.zymed.com).

Results and discussion

Dilution, mixing and incubation

Injection of a dye into a nonreactive colorless carrier stream is an established technique for the characterization of a flow injection system,[13] since it identifies the critical parameters for assay optimization. The sample volume $S_{1/2}$ is the volume injected to reach 50% of the steady state signal and was found to be approximately 6 µL for the CSPU furnished with 500 µm channels. This value was found from the leading edges of the response curves, obtained by injecting bromothymol blue (BTB) in the range 2.0–80 µL. In contrast, the slopes of the trailing edges of the response curves increased with increasing volume of the injected dye. Thus, when a steady state response

level was reached at $D = 1$ ($D = C_o/C_{max}$ where C_o is the original concentration of injected dye and C_{max} is the dye concentration in the flow cell at peak maximum), the $S_{1/2}$ of the trailing edge of the 80 µL zone was 45 µL. The difference between $S_{1/2}$ of the leading and trailing edges is caused by the difference in the length (and volume) of the channel through which the molecules of dye must travel. Thus the dye within the leading section of the zone travels only a short distance between port #2 and the flow cell, while the dye from the trailing edge of the same zone goes first by flow reversal into the holding coil and then through the same path back into the valve, *via* port #2, and into the flow cell. Clearly, the longer the flow reversal, the larger the dispersion and the $S_{1/2}$ value. Computer control allows variability in the distance travelled, an important advantage as it offers a wide range of sample processing options.

To increase dilution and mixing, all that is needed is to increase the magnitude of the flow reversal and the distance travelled. This is achieved by injecting an additional volume of solution, that will push the sample solution deeper into the holding coil. The influence of this 'spacer' zone is shown in Fig. 4, where a series of taller peaks is obtained by injecting increasing volumes of a dye without the spacer zone, while the series of shorter and more symmetrical peaks shows the influence of a 20 µL spacer zone that was injected following dye injection. Thus, while the injection of dye into the flow cell yields an $S_{1/2}$ value of 5 µL, the use of a 20 µL spacer zone increases $S_{1/2}$ to approximately 23 µL, consistent with the increased flow path through which the dye must travel. (Steady state value for $D = 1$ was measured at $A = 1.25$.) This experiment demonstrates, that by increasing the 'spacer' volume, dilution increases and mixing is promoted, as seen from the peak shapes, which change from an asymmetrical to a nearly Gaussian shape (see also Figs. 5 and 6).

Reagent based assays require mixing of the analyte with one or several reagents in an appropriate sequence. Dye injection experiments, which simulate the mixing of sequentially injected zones (Fig. 5), show the influence of the injection sequence and spacer volume on the peak overlap of sample and reagents. To visualize the overlap, three experimental runs, each comprising two injection sequences, are shown superimposed in Fig. 5. The first injection sequence (#1) was recorded as three injected zones (A, B, C) directed into the flow cell through the shortest possible route (channel volume, 5 µL). In the second injection sequence (#2), the three zones were first pushed upstream using a 20 µL spacer zone (shown as shaded box in Fig. 5), before being directed by flow reversal into the flow cell. Since each of the three experimental runs comprised three 10 µL injections, of which only one (A in the first run, B in the second and C in the third) used a dye (the other two injections were of colorless
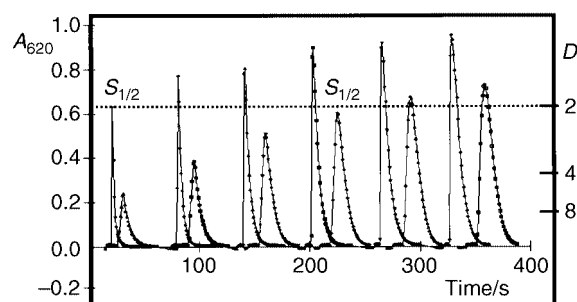

Fig. 4 Influence of sample and spacer zone volumes on $S_{1/2}$ and dye dilution. Response curves obtained by injecting bromothymol blue (200 ppm BTB) without (initial peaks) and with (second peaks) the aid of a 20 µL spacer zone. Responses were monitored in absorbance mode in a flow cell configured as shown in Fig. 3A with a light path of 1.5 mm. Injected BTB volumes are 5, 10, 15, 20, 25 and 30 µL; flow rate, 4 µL s^{-1}; carrier and spacer solution, borax buffer (pH 9) (for details, see text).

carrier solution), the overlap of the individual zones is resolved. Since only zone 'C' is overlapped by both zones 'B' and 'A', it follows that the sample zone should always be injected first into the holding coil, followed by reagents (A, B). By injecting the spacer zone, mixing of the sample with reagents is further promoted.

Incubation time and stopped flow

The most efficient way of providing incubation time is to stop the flow following the initial mixing of the sample and reagent zones.[13,14] While, in principle, the composite analyte/reagent zone can be held within the holding coil, it is more efficient and informative to provide the incubation time while the analyte is still within the flow cell, since this allows the rate of reaction to be monitored. The dye injection experiment (Fig. 6) gives a clue to the optimization of assay parameters. Following sample (10 µL of a dye) and reagent (2 times 10 µL of carrier solution from ports #6 and #4) injection the spacer zone was injected (20 µL of carrier from port #3), pushing the sequenced zones upstream into the holding coil. Then the flow was reversed and the composite zone was pushed through the valve *via* port #2 and
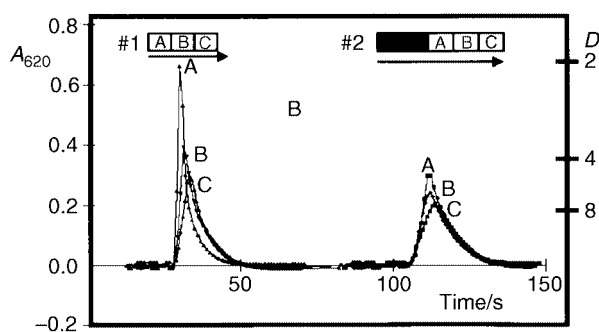
Fig. 5 Dye injection (BTB) shows the degree of overlap of 'sample' (C) and 'reagent' zones (A, B) without spacer (run #1) and with 20 µL spacer zone (run #2, shaded box) pushing zones A, B, C upstream (as indicated by arrows) into the holding coil. Note the complete zone overlap and peak symmetry caused by the use of the spacer zone. (Experimental conditions the same as in Fig. 4; injected volumes of A, B, C zones, 10 µL; for details, see text).
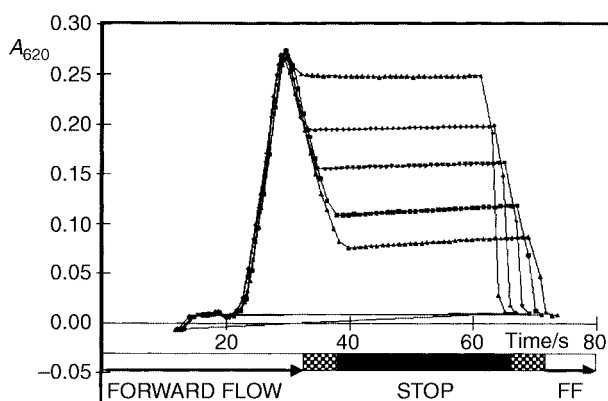
Fig. 6 Stopped flow experiment obtained by dye injection. The five experimental runs show how stopping the flow provides an incubation time for reaction rate measurement. The composite 'sample' (dye, BTB) and two 'reagent' zones were injected into the holding coil by using a 20 µL spacer zone, (same experimental conditions as run #2 shown in Fig 5). When the flow was reversed, the composite zone was pushed downstream and inserted into the flow cell by dispensing an increasing volume of carrier (55, 65, 75, 85 and 95 µL) for each of the five experimental runs, followed by a 30 s stop flow period.

into the flow cell, where the flow was stopped for 30 s. Finally, the flow was resumed at an accelerated rate to wash out the system. Five experimental runs, shown overlain in Fig. 6, demonstrate that the sensitivity of the reaction rate measurement will depend on the volume of solution used to push the composite analyte zone into the flow cell.

The following conclusions can be drawn. (i) The sensitivity of measurement will increase *linearly* with the volume of sample injected until the $S_{1/2}$ value is reached. A further increase in the sample volume is wasteful and may lead to insufficient analyte/reagent mixing. Analyte dilution and mixing are promoted by increasing the $S_{1/2}$ value through the use of spacer and reagent volumes. (ii) The analyte sample should always be injected first, followed by reagents and finally by spacer zone. For a higher sensitivity, the spacer zone can be replaced with larger reagent zones (see Table 1). (iii) Stopping the flow when the highest concentration of analyte passes through the flow cell allows for reaction rate measurement. The highest sensitivity is achieved when the volumetric displacement by reversal flow is equal to the volumetric sum of the reagents and spacers used.

The dye injection experiments show that the spatial and temporal resolution of sample processing in the 'lab-on-valve' system operated in a µSI mode is highly repeatable, even if carried out several days apart. The first peak in Fig. 5 and the second peak in Fig. 4 have identical absorbances at the peak maxima. A similar repeatabilty is shown by comparing peak 'A' in the second run in Fig. 5 with the peak maxima in Fig. 6.

Two reagent stopped flow assay

Phosphate assay. Assay of orthophosphate, based on the formation of phosphomolybdenum blue,[13,15] has been selected as an example of a reagent based assay, since it is one of the most frequently performed assays by flow injection,[16] and also because it presents several difficulties that can only be avoided through a precise control of experimental conditions. First, the product of the assay (molybdenum blue) is a colloidal suspension, not a true solution. Next, the reagents used are not compatible when premixed, and need to be added in a sequence that allows the phosphomolybdate to be formed first, followed by reduction to a blue product for spectrophotometric monitoring (at 712 or 880 nm.[15]) The reduction is slow, yet it can be catalyzed (by Sb-tartarate), and the rate of reaction is pH dependent. Following the previously outlined rules, an experimental protocol was designed to inject sample (10 µL 30 ppm P solution, port #5), molybdate reagent (10 µL port #6) and reducing solution (ascorbic acid 30 µL port #4) into the holding coil (larger volume of ascorbic acid substitutes for the spacer zone). The composite analyte/reagent zone was then pushed downstream into the flow cell and stopped for 30 s. The flow cell was configured for absorbance measurement with an extended 7 mm flow path (Fig. 3B). Two consecutive sequences, each comprising five experimental runs, are shown superimposed (Fig. 7), and the influence of the positioning of the composite analyte/reagent zone within the flow cell on the slope of the reaction rate curve is seen. The positioning is controlled by volumetric control of the flow reversal (45, 50, 55, 60 and 65 µL) in analogy with the stopped flow experiment shown in Fig. 6. Blue product formation through time causes the absorbance to increase during the stopped flow period, yielding reaction rate curves. Note that, as the analyte concentration decreased along the trailing edge of the dispersed analyte peak, the slope of the reaction rate curves decreased accordingly.

The phosphate assay was carried out by a stopped flow protocol and optimized with aid from the previous experiments (Table 1). The salient features of the protocol are: sample volume, 4 µL; molybdate reagent, 10 µL; ascorbic acid, 15 µL;

push into the flow cell, 30 μL; stopped flow period, 40 s. Since, in this experiment, the injected sample volume was very small, an additional precaution was taken to avoid carryover by flushing out the sampling port (#5) by 5 μL of the *next* sample ('sample port washout' Table 1). Absorbance was measured at 855 and 712 nm using a flow cell with a light path extended to 7 mm (Fig. 3B). Each sample was injected in duplicate and the calibration curve was constructed using peak maximum measurements, since the stopped flow period was identical for all the measurements (Fig. 8). Samples in the range 0–40 ppm P yielded a calibration curve with a blank value of 2 ppm P. The slope of the calibration curve at 855 nm was 37 ppm P/0.1 A and at 712 nm was 56 ppm P/0.1 A. The detection limit (estimated at twice the blank value) is 4 ppm of P in a 4 μL sample volume, corresponding to 16 ng P. Since the blank is probably due to traces of P in the reagents used, and since the injected volume can be increased up to five times (before $S_{1/2}$ defined by this fluidic protocol is reached, compare Fig. 5), it can safely be estimated that increasing the injected sample volume five times would improve the detection limit below 5 ng of P, followed by a corresponding increase in sensitivity. The concentration presented in Fig. 8 was selected to cover the range compatible with the monitoring of fermentation processes. Finally, it is essential to note that it was necessary to include a surfactant into the carrier stream (see Reagents), since the colloidal phosphomolybdenum blue gradually deposits on the channel walls and within the flow cell, causing a severe baseline drift.
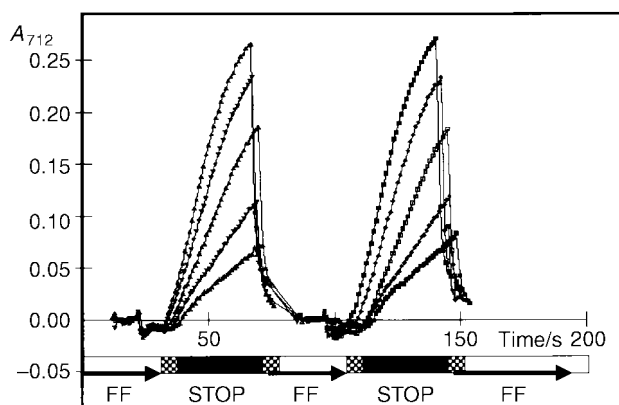

Fig. 7 Optimization of stopped flow reaction rate measurement of orthophosphate, showing two experimental sequences, comprising five superimposed experimental runs, obtained by injecting 10 μL 30 ppm P solutions followed by appropriate reagent zones into the holding coil. The positioning of the analyte zone in the flow cell for reaction rate monitoring was controlled by volumetric control of the carrier solution that was used to push the reacting zone from the holding coil downstream (45, 50, 55, 60 and 65 μL). Formation of molybdenum blue was monitored in absorbance mode in a flow cell with an extended light path (7 mm).
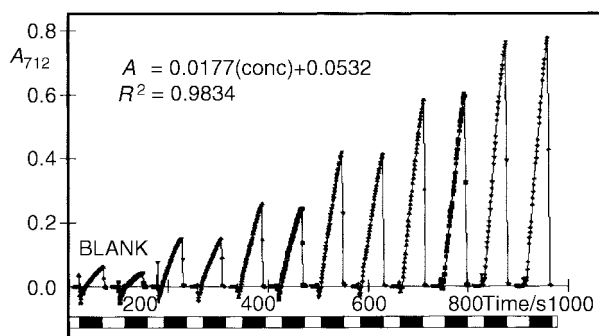

Fig. 8 Stopped flow spectrophotometric assay of orthophosphate in the range 0–40 ppm P. Injected sample volume, 4 μL; molybdate, 10 μL; ascorbic acid, 15 μL. Detection limit, 16 ng of P. All samples injected in duplicate. For details, see text and Table 1.

Enzymatic assay of protease activity. This was based on the enzymatic degradation of an artificial fluorogenic substrate (7-[*N*-α-succinyl-L-alanyl-L-alanyl-L-(phenylanyl)amino]-4-methylcoumarin) that consists of a fluorophore (7-amino-4-methylcoumarin, AMC) covalently attached to a tripeptide substrate (AAF). As the fluorophore is released from the tripeptide by proteolytic action, fluorescence increases and can be measured at 450 nm.[17] The protease, savinase, is an alkaline endoprotease of serine type, industrially produced as a detergent additive, and its properties and methods of assay are well described.[12,17] The experimental protocol was adjusted to inject enzyme (15 μL), followed by substrate (30 μL) and flow reversal of 40 μL, after which a stopped flow period of 60 s was used to monitor the increase in fluorescence. The flow cell was configured for fluorescence monitoring (Fig. 3C). The enzymatic activity was assayed from the slope of the reaction rate curve in the range 10 to 0 KNPU units. (Fig. 9) The calibration is in good agreement with previously published results.[12,17]

Bead injection based bioligand interaction assay. All assays based on liquid–solid interactions benefit from precise fluidic control, and therefore their processing within the CSPU results in their miniaturization and improved reliability. Bead injection[7] uses suspended beads as carriers for reactive groups or reagents. The bead suspension is injected into the flow channel, where the beads are trapped and perfused by analyte solutions, buffers and/or auxiliary reagents. (Bio)chemical reactions taking place at the bead surfaces can thus be monitored in real time, either directly on the solid phase or by monitoring the eluting liquid phase. In this work, the monitoring of beads in the jet ring cell configuration was performed by absorbance measurement (Fig. 3D) or by fluorescence (Fig. 3E).

Bioligand interaction of recombinant protein G with anti-mouse IgG-labeled FITC has been monitored by absorbance and fluorescence spectroscopy, using identical experimental protocols for fluorescence emission measurement at 525 nm (Fig. 10) and UV absorbance of IgG protein at 262 nm, with the aim to compare the sensitivities of measurement of tagged and nontagged sections of IgG-FITC molecule in the multipurpose flow cell. The experimental protocol comprised the following steps: (i) aspiration of bead suspension (4 μL) from the syringe attached to port #6 into the holding coil; (ii) flow reversal (50 μL) to transfer and pack beads in the flow cell; (iii) aspiration of 25 μL sample solution (port #5) into the holding coil; and (iv) flow reversal at a flow rate of 1 μL s⁻¹, bringing the sample zone in contact with the protein G–Sepharose beads, resulting in the capture of IgG-FITC on the bead surfaces. A carrier stream of HBS buffer (pH 7.4) was used in all experiments. For fluorescence measurement of the fluorescent tag, the concentrations of injected IgG-FITC were 4.0, 10.0, 25.0, 50.0 and 100 μg mL⁻¹ (Fig. 10). For absorbance measurement of the protein
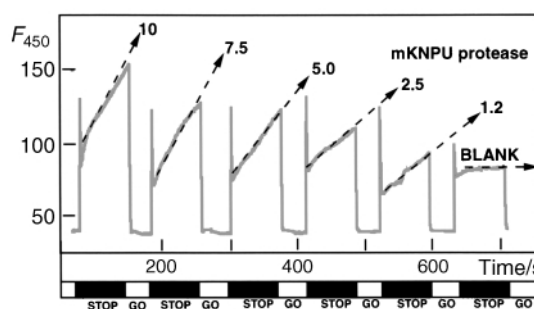

Fig. 9 Fluorometric assay of the enzymatic activity of a protease based on the degradation of an artificial fluorogenic substrate. The reaction rate was measured by the increasing fluorescence of AMC released during the stopped flow period. Injected enzyme volume, 15 μL.

part of IgG-FITC, the concentrations of the injected biomolecule were 10, 25, 50 and 100 µg mL^{-1}. During the exposure period, the fluorescence and absorbance increased as the IgG-FITC molecules accumulated on the bead surfaces. Since the IgG–protein G interaction is very strong, the IgG remains firmly attached during the following washout period, as seen from the constant level of response. At the end of the measuring period, beads were removed from the jet ring cell by a fast flow reversal (50–25 µL s^{-1}) into the holding coil, and then discarded from the system *via* port #1.

Taking 0.004 A and 3.0 F units as a minimum reliably detectable signal, the detection limit for absorbance measurement was 10 µg IgG mL^{-1} (total injected amount, 200 ng) and for fluorescence measurement was 5 µg IgG mL^{-1} (total injected amount, 100 ng). For absorbance measurement, linear regression was valid ($y = 0.0003x + 0.0088$, $R\exp2 = 0.9914$); for fluorescence, a nonlinear fit applied ($y = -0.0019x \exp 2 + 0.6686x + 0.6855$, $R\exp2 = 0.9981$). There is, however, room for improvement of the detection limit and sensitivity. The significance of this experiment is that it shows that the jet ring cell integrated in the µSI system provides highly repeatable data. This can be seen in Fig. 10, showing two experimental runs superimposed. Also a novel construction of the jet ring cell, where the spent beads are discarded by a swift flow reversal, is less mechanically complex than the previous jet ring flow cell design, based on magnetically actuated opening of the circular gap through which the beads were discarded by a forward flow.[7] It is the short channel between the flow cell and port #2 that makes the use of flow reversal practical. The long conduit between the valve and the flow cell used in previous constructions was prone to retain some of the spent beads if flow reversal was used.

Conclusions

The approach to microfluidic assays described in this work is conceptually different from the methodology that is currently applied to microfabricated devices designed for automation of reagent based assays. While application of continuous forward flow is a necessity when performing chromatographic or electrophoretic separations, reagent based assays are better performed using discontinuous multidirectional flow that affords unprecedented versatility in the sequencing of sample handling operations. While the use of continuous forward flow (on which all current µTAS systems are based[1–3]) necessitates microfabrication of a dedicated sequence of components (mixing Tees, mixers, reactors, flow cell, *etc.*) suited to a single type of assay, this work, in contrast, shows how flow programming can be used to allow for multiple uses for a single device. Nicknamed 'lab-on-valve', this CSPU operates in the present form at µL level, yet it can be downscaled, if desired, to an upper nL range by using tubing of smaller id and a microsyringe pump capable of slower flow rates. This raises the question of how far downscaling of analytical systems should go. While the advantages of microminiaturization of chromatographic separations have been well documented,[1–3] and downscaling to a sub-nL level is a necessity for certain applications (single cell analysis, electrospray MS[18]), downscaling of the vast majority of reagent based assays, below µL level, seems to create more difficulties than benefits. Assuming that pumping, valving and mixing could be carried out within microfabricated devices reliably, two obstacles remain: the introduction of real life samples into the 'lab-on-chip', and washing out the working channels after the assay has been completed. (Discarding chips after a single use does not seem to be an economically sound proposition.) Indeed, sample introduction and system washout are the most wasteful operations. In real world applications, the distance between the sample container (sample changer) and sampling port necessitates the use of a conduit, the volume of which may well be several microliters, requiring even greater volumes of sample solution for reliable washout between injection of analytes of different compositions ('sample port washout', Table 1). Reliable washout of CSPU requires, as a rule, a minimum of five times its internal volume. Although washout volumes used in this work were somewhat excessive (Table 1, 'prewash' and 'system washout'), and could be reduced, the amount of waste generated per assay will remain, realistically, at about 250 µL. While this is a significant improvement compared to the present rate of waste generation in a routine laboratory, it is questionable whether further reduction of waste to micro- or submicroliter level is desirable.

Thus the true advantages of using microfluidics for reagent based assays are the compactness of the apparatus, automation of all steps of the experimental protocol and, last but not least, the integration of all manifold components into a permanent rigid structure that enhances the repeatability of sample processing operations.

As this paper is being written, several applications of the µSI technique are being explored. For a multireagent assay, the flow through the central port of the CSPU (P2, Fig. 2) is connected to a second syringe pump,[9] allowing the mixing of multiple zones which are first stacked into two holding coils and then merged by simultaneous pumping through the central port where their comixing takes place. Use of a double pumped CSPU offers the versatility of a wider range of flow rates and sample handling sequences. Another interesting feature of CSPU construction is that it can also accommodate renewable microcolumns. By retrieving tubing towards the periphery of the CSPU (as shown in Fig. 2, port #1), a cavity is formed (1.5 mm id, up to 15 mm long) in which beads can be injected, retained and flushed out after use. In this way, chromatographic materials can be moved to strategic locations within the CSPU for analyte preconcentration, matrix removal and analyte elution. Such a configuration of lab-on-valve is presently being tested as a front end sample processing unit for graphite furnace AA, electrospray MS and capillary electrophoresis. Indeed, we are only at the beginning of research that will enable us to exploit the versatility and power of microfluidic manipulations based on the SI technique.
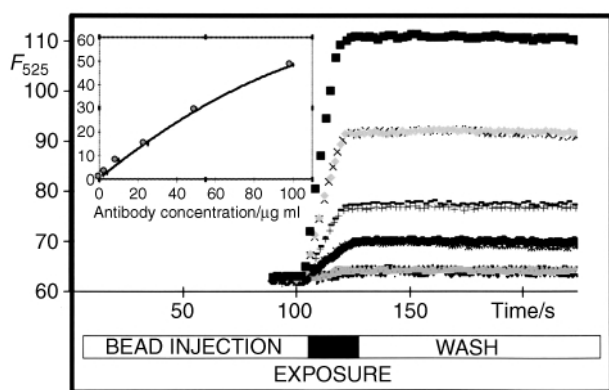

Fig. 10 Fluorescence monitoring of adsorption of anti-mouse IgG-FITC molecules on the surface of Sepharose 4B–protein G beads. Injected bead volume, 4 µL; injected sample volume, 25 µL; flow rate, 2 µL s^{-1}; carrier, HBS buffer. Flow cell configuration, Fig. 3E; tungsten light source and cut-off filter at 505 nm.

Scampavia for critical comments and FIAlab Instruments for material support.

References

1 *Micro Total Analysis Systems '98*, ed. J. D. Harrison and A. van den Berg, Kluwer Academic Publishers, Dordrecht, Netherlands, 1998.
2 *Microsystem Technology in Chemistry and Life Sciences*, eds. A. Manz and H. Becker, Springer Verlag, Berlin, 1998.
3 M. Freemantle, *Chem. Eng. News*, 1999, Feb. 22nd, 27.
4 D. C. Duffy, H. L. Gillis, J. Lin, N. F.Sheppard Jr. and G. L. Kellogg, *Anal. Chem.*, 1999, **71**, 4669.
5 W. E. van der Linden, *Trends. Anal. Chem.*, 1987, **6**, 37.
6 J. Ruzicka, *Analyst*, 1994, **119**, 1925.
7 J. Ruzicka and L. Scampavia, *Anal. Chem.*, 1999, April 1st, 257A.
8 J. Ruzicka, *Flow Injection*, E-monograph on CD-ROM, self-published at jarda@flowinjection.com, 1999.
9 J. Ruzicka and E. H. Hansen, *Anal. Chem.*, 2000, **72**, 212A.
10 J. Ruzicka, C. H. Pollema and K. M. Scudder, *Anal. Chem.*, 1993, **65**, 3566.
11 J. Ruzicka and A. Ivaska, *Anal. Chem.*, 1996, **68**, 5024.
12 *Savinase Product Sheet*, Detergent Enzyme Division, Novo-Nordisk Industries, Copenhagen, 1986.
13 J. Ruzicka and E. H. Hansen, *Flow Injection Analysis*, Wiley, New York, 2nd edn., 1988.
14 E. B. van Akker, M. Boss, A. van den Berg and W. E. van der Linden, in *Micro Total Analysis Systems '98*, ed. J. D. Harrison and A. van den Berg, Kluwer Academic Publishers, Dordrecht, Netherlands, 1998, p. 335.
15 Z. Marczenko, *Separation and Spectrophotometric Determination of Elements*, Ellis Horwood Series in Analytical Chemistry, John Wiley, New York, 2nd edn. 1986, ch. 39.
16 S. Chalk, *Flow Analysis Database*, at http://www.fia.unf.edu/fad/fad.html.
17 Y. Kanaoka, T. Takahashi, H. Nakayama and K. Tanizawa, *Chem. Pharm. Bull.*, 1985, **33**, 1721.
18 S. Ekstrom, P. Onnerfjord, J. Nilsson, M. Bengtsson, T. Laurell and G. Marko-Varga, *Anal. Chem.*, 2000, **72**, 286.