Controlled microfluidic reconstitution of functional protein from an anhydrous storage depot

Elena Garcia,* Jared R. Kirkham, Anson V. Hatch, Kenneth R. Hawkins and Paul Yager
Department of Bioengineering, University of Washington, Box 352141, Seattle, WA 98195-2255, USA; Fax: (206) 616-1984; Tel: (206) 616-1928

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A novel method has been developed for preserving molecules in microfluidic devices that also enables the control of the spatial and temporal concentrations of the reconstituted molecules within the devices. In this method, a storage cavity, embedded in a microchannel, is filled with a carbohydrate matrix containing, for example, a reagent. When the matrix is exposed to flowing liquid, it dissolves, resulting in the controlled reconstitution and release of the reagent from the cavity. The technique was demonstrated using two different model systems; the successful preservation and controlled release of β-galactosidase was achieved. This method has possible applications for simple point-of-care drug delivery and immunoassays, and could be used to pattern the surfaces of microchannels. More broadly, this preservation and controlled release technique can be applied where the preservation and/or spatial and temporal control of chemical concentrations is desired.

Introduction

Microfluidic devices can perform a wide variety of functions. For instance, they have been used to separate molecules,1–3 perform assays,4,5 and genotype.6,7 Some microfluidic systems have successfully combined multiple functions.8 This technology has been reviewed recently.9–12

A major liability of most current microfluidic devices is that they require substantial support equipment outside of the microfluidic components themselves. This added bulk would severely limit the applicability of microfluidics to point-of-care applications. One way to reduce the bulk, particularly for single-use devices, would be to store all required reagents within the devices. The incorporation of preserved reagents into lab-on-a-chip systems would simplify operation, but many biochemicals do not store well in solvents. On-chip dry reagent preservation would not only provide greater portability, but would also allow long-term storage of all required chemicals and biochemicals in uncontrolled environmental conditions, such as the high temperatures of the tropics. This would increase the lifetime of dry-reagent-based devices, decrease reagent waste, simplify their operation, and make them more portable and robust.

Methods of stabilizing proteins, vaccines, and organs for transplantation using trehalose (α-D-glucopyranosyl α-D-glucopyranoside) are widely used in industry.13 Many proteins have been preserved by drying them in the presence of trehalose,13–20 and its protein stabilization characteristics have been extensively studied.13,20,21 Because trehalose is a non-reducing disaccharide, it is thought to be largely due to the substitution of the waters of hydration of the protein by the sugar molecules during drying, allowing maintenance of the native state of the protein.21

Our laboratory is developing tools for point-of-care instrumentation for monitoring concentrations of analytes in biological fluids. One aim is to create disposable polymeric devices that are capable of carrying out several quantitative bioassays in parallel, which may require devices with channel widths on the order of 1 μm. This report describes preliminary work on the storage and preservation of biomolecules in microfluidic devices within cavities in one wall of a microchannel. The cavities are both storage depots and a microfluidic device for the spatially and temporally controlled dissolution of the stored chemicals into the microchannel during flow. The level of controlled release afforded by the incorporated microcavities would be difficult to achieve by preserving the reagents directly on microchannel surfaces. Controlled spatial and temporal dissolution of reagents from cavities in microchannels can be used to deposit molecules on the surfaces of microchannels, as part of a homogeneous phase assay, or as a general way to transport reagents in lab-on-a-chip systems. Using two model systems, we demonstrate controlled spatial and temporal reagent dissolution in a microchannel.

Results and discussion

Microchannels were created that contained a storage cavity along one wall. These simple microfluidic devices were constructed according to the schematic shown in Fig. 1. Before a device was assembled, the cavity in the bottom layer was filled with a concentrated solution of trehalose and dextran that contained a reagent of interest. Trehalose was incorporated into the storage matrix because of its preservation properties. Dextran served to increase the viscosity of the material in or dissolving from the cavity, thereby slowing its overall rate of release. The cavity, filled

Fig. 1 Generic design of microfluidic test devices. By placing a channel layer (middle) between an inlet/outlet layer (top) and a cavity layer (bottom), microchannels were created that contained a storage cavity in one wall of the microchannel. This figure is not to scale. The widths of the channels (x-dimension) ranged from 2 to 2.5 mm. The height of the channels and the cavities (y-dimension) ranged from 100 to 250 μm. Both cylindrical- and rectangular-shaped cavities were created to hold the dried matrix. In the x-y plane, the diameters of cylindrical cavities were 250 μm, and the sides of rectangular cavities were 500 μm.
with the carbohydrate and reagent solution was allowed to dry, producing a storage matrix. Then, the device was assembled creating a microfluidic channel with a filled storage cavity along one wall. Fluid was pumped into the assembled device, causing the dissolution of the storage matrix and the reagent from the cavities.

From first principles it is clear that release of material from the cavity and its subsequent transport downstream may be controlled by parameters that change the material’s diffusive and convective transport. These parameters include channel and cavity dimensions, cavity shape, fluid flow rate, and the chemical composition of the carbohydrate matrix used for preservation. The controlled plume of molecules produced by dissolution from the storage cavities could be exploited to deposit molecules on a downstream surface (Fig. 2A). For example, primary or secondary antibodies could be deposited for an immunosassay, or alkanethiols could be deposited to create self-assembled monolayers. Alternatively, the technique could be used directly as part of a homogeneous phase assay (Fig. 2B) such as T-sensor based assays like the diffusion immunoassay developed in this laboratory. We report on the application of the later configuration.

**Controlled dissolution of the storage matrix**

To demonstrate the ability of the storage matrix to produce a controlled plume of redissolved molecules, a cylindrical storage cavity 250 µm in diameter and 200 µm deep was formed in the wall of a microchannel (Fig. 1). The cavity was filled with a concentrated solution of trehalose and dextran (in a ratio of 3:2 by weight) that had been spiked with fluorescein as an indicator. The fluorescein retained its fluorescence after the matrix had been stored under a laminar flow hood for several days. When the cavity was exposed to flowing aqueous solution, the resulting dissolution plume downstream from the cavity could be monitored quantitatively using fluorescence imaging (Fig. 3).

Fluorescence images were taken every 30 s for 630 s during the dissolution of the storage matrix from the cylindrical cavity (Fig. 3) and were used to find relative indicator concentrations at a location ~ 520 µm downstream from the center of the cavity. Relative fluorescence intensities of the indicator were found for each of the time points as shown in Fig. 4. During the first ~ 120 s, there was a rapid drop in the intensity of the dissolution plume to ~ 40% of the maximum value. From 200 to 600 s, the measured intensity was more stable, dropping to ~ 20% of its maximum value. It was still possible to detect the dissolution plume after 10 min. After approximately 15 min, no fluorescein was detected in the cavity. These data demonstrate that the concentration of a dissolving chemical can be controlled temporally using this technique.

**Controlled dissolution of a preserved enzyme in the storage matrix**

The enzyme β-galactosidase (β-gal) was chosen as a model enzyme for study of preservation and reconstitution from storage depots. When the small molecule resorufin-β-galactoside (RBG) is used as a β-gal substrate, the essentially non-fluorescent substrate is cleaved by β-gal to yield the products galactose and the fluorescent dye resorufin. Cleavage of RBG by β-gal is a single-step process that follows Michaelis–Menten kinetics. The simple enzyme kinetics of the β-gal–RBG system, along with the fact that RBG is fluorescent, makes this an attractive model system.

Rectangular storage cavities were filled with β-gal in a concentrated solution of trehalose and dextran, dried in an oven for 7 days at 55 °C (± 5 °C), then placed in a desiccator for an additional 7 days. The β-gal-loaded storage cavities were subsequently incorporated into microchannels and were exposed to flowing.
solutions of 8.2 μM RBG in buffer. The resulting enzymatic product, resorufin, was detected using fluorescence imaging. The presence of resorufin was used to indicate successful dissolution of active enzyme from the cavity (Fig. 5). Fluorescence was not observed when control cavities containing the preservation matrix without enzyme were exposed to the solution of RBG. It took approximately 50 s for the β-gal in the storage cavities to completely dissolve.

The preservation of the enzyme in the wells was examined after filled cavities had been prepared for the dissolution experiments as described above. By measuring the percentage of enzyme activity remaining in the cavities using the initial velocity method, it is estimated that 30–80% of the initial enzymatic activity remained during the dissolution experiments. The large range in the estimated activity of the enzyme likely resulted from the large variation in the volumes of enzyme solution (150 ± 75 nL) placed in the cavities.

Comparison of cylindrical and rectangular cavities

To compare dissolution from cavities with circular and square openings into the flow channel, the indicator profiles transverse to flow at distances downstream from the cavities were measured from images (Fig. 6). Since the two systems had different dimensions and used different indicator molecules, fluorescent intensities and geometric dimensions were normalized.

While it is not a perfect comparison, a trend is clear; the maximum concentrations of indicator for cylindrical cavities were positioned directly downstream from the center of the cavities, while the maximal indicator concentrations generated by the enzyme released from rectangular cavities were found approximately downstream from the corners of the cavity. Profiles for rectangular cavities were often not symmetric. There are two possible explanations for this. It was not always possible, in the experiments, to align the flow axis parallel to one side of the square opening of the cavity. Also, irregularities in the initial shape of the plug of dry matrix would lead to long-term irregularities in the downstream profiles of indicator molecules.

Comparison of the dissolution trends from the two types of cavities is complicated by the nature of the indicator molecule; the indicator molecule for the rectangular cavities was the product of an enzymatic reaction, while the indicator for the cylindrical cavities was released directly from the storage matrix. For the enzymatic reaction, the concentration of indicator molecules depended on the mass transport of the substrate to regions containing enzyme and on the rate of product formation. Also contributing to the shape of the released plume are the flow velocity vectors in the depots, which are different for the cylindrical- and rectangular-shaped cavities.

Both the nature of the indicator molecule and the flow velocity vectors inside the cavities during reagent release contributed to the differential concentration profiles found (Fig. 6). Further modifications of the shape of the cavity or plug of matrix should allow control of the shape of the plume of dissolving chemicals. To make such studies quantitative and comparable with simulations, future studies will employ controlled volumes of matrix in the cavities.

Conclusions

The storage of reagents in dry form is a critical requirement for the development of such microfluidic devices as point-of-care diagnostic systems. The “structured” dissolution plume of reconstituted reagents can subsequently (and rapidly) be made uniform prior to use by following the storage cavity with any of several designs of microfluidic mixers. However, the structure of the dissolution plume itself could be useful. For example, in surface patterning applications, there may be a need for the reconstituted reagents to be present in non-uniform concentrations across the length, width, or height of a microchannel. Controlling the dissolution of chemical species or particles from an anhydrous storage depot in a microchannel is a novel way to control the spatial and temporal concentrations of such species in microchannels. This naturally can lead to solutions that are non-uniform in predictable ways.

Control of the temporal concentration of a solute dissolving from a dry storage depot for one to over 10 min has been achieved. The enzyme β-gal has been preserved in the anhydrous storage depots, placed in a microchannel, reconstituted, and released in active form when exposed to flowing solution containing its substrate RBG. The concentration of the dissolved anhydrous storage depot indicator transverse to flow downstream from the depot had one maximum for cylindrical storage cavities and two for rectangular storage cavities. Changing the shape of the storage cavity is one way spatial concentrations of reagents in a microchannel can be controlled. Other means of control, including the flow rate or the fluid in the channel, the channel and cavity dimensions, and the composition material in the cavities, are currently being explored.

Experimental protocol

Computational fluid dynamic simulations

The two-dimensional simulation used to produce Fig. 2 was performed using FEMLAB (COMSOL Inc., Burlington, MA). The non-dimensional Navier–Stokes equation was employed to sim-

Fig. 5 Fluorescence image of reconstitution of a functional enzyme as demonstrated by the production of the product of that enzyme in a flowing stream of buffer. The storage cavity shown (top view) was 500 × 500 × 100 μm deep. As the storage cavity containing dried β-gal was exposed to RBG solution, β-gal was released by dissolution of the matrix in the cavity. Once in solution, the β-gal cleaved RBG to produce the fluorescent product resorufin (which was the only fluorescent species in the image). Flow was from left to right.

Fig. 6 A comparison of the indicator concentrations along a line perpendicular to the flow and in the plane of the surface of storage depot for circular and square openings into the flow channel as measured ~ 500 μm downstream from the cavities. Fluorescence intensities were normalized by dividing the intensities by the maximum intensity for each system. Differences in the size of the cavities were accounted for by dividing the distance in the transverse flow position by the diameter or side length for cylindrical- and square-shaped cavities, respectively. Square cavities consistently produced bimodal concentration profiles.
ulate fluid flow in the channel, while the non-dimensional convection–diffusion equation was used to simulate the dissolution of a chemical species from the cavity during flow. The Peclet number was 100, defined as the average velocity times a characteristic length of 100 μm divided by the viscosity of pure water. The top and bottom surfaces of the channel (the surfaces parallel to the x-axis in Fig. 2) and the exposed surface of the dissolving matrix were given no-slip boundary conditions. The boundary that simulated the channel inlet (left channel wall parallel to the y-axis in Fig. 2) had a parabolic velocity profile, while the simulated outlet (right channel wall parallel to the y-axis in Fig. 2) had a straight out boundary condition, i.e. the pressure there was set to zero and the dot product of the vectors tangent to the boundary and velocity vectors were zero. The boundary conditions for concentration were set to zero everywhere, except on the line simulating the exposed surface of a material dissolving from the cavity (Fig. 2), which was set equal to one. The boundary line representing the bottom of an exposed surface in the cavity with a concentration of one was not changed during the simulation to model a steady state. Similar models are under development to explore various parameters important to the dissolution of a chemical species from a cavity in a microchannel.

Reagents and reagent transfer

Cylindrical cavities were filled with a fluorescein (Sigma, St. Lois, MO)-spiked solution of 30% (w/) trehalose (Sigma, St. Lois, MO) and 20% dextran (average molecular weight 68 000; Sigma, St. Lois, MO) (w/w) in a buffer solution of 0.1 M Tris HNO₃, 0.1 mM MgCl₂, and 2 mM KCl, pH 7.8 (Sigma, St. Lois, MO). Volume was transferred into the cavities using stretched glass capillary tubes attached to pipettes. The matrix material in the cavities was dried by placing the open PDMS surfaces in a laminar flow hood for 48 h. Deionized water was used to dissolve the storage depots in the assembled devices.

Rectangular cavities were filled with a solution of 1.95 mg mL⁻¹ of β-gal from Escherichia coli (Sigma, St. Lois, MO) in a buffer solution of 10 mM sodium phosphate, 0.138 M NaCl, and 2.7 mM KCl, pH 7.4 (Sigma, St. Louis, MO) containing 13.3% trehalose and 20% dextran (w/w). Enzyme solution was transferred manually into the cavities using a 457 μm diameter hydrophobic pin (V&P Instrument Company, Vernon Hills, IL) that was molded into the PDMS during curing. The bottom layer (Fig. 1) of the device was fabricated as described above for the cylindrical cavities, except the cavities were rectangular with sides of 500 μm and depths of 100 μm.

Flow cells

Flow cells for devices containing cylindrical cavities were made using a microscope cover slip with drilled inlet and outlets as a top layer as described in Hatch et al.³ (Fig. 1). A middle layer (Fig. 1) was made of 200 μm thick Mylar coated on both sides with 25 μm thick pressure-sensitive adhesive (Fraylock, Inc., San Carlos, CA), through which channels had been cut using a CO₂ laser (Universal Laser Systems, Inc., Scottsdale, AZ). A bottom layer (Fig. 1) of polydimethyl siloxane (PDMS) (Sylgard 184 Dow Corning Corporation, Midland, MI) that contained the cylindrical storage cavity was created using soft lithography methods.²⁸ The cylindrical cavities were 250 μm in diameter and 200 μm deep. The width of the microchannel (the z-dimension in Fig. 1) was 2 mm. The large width of the channel minimized transport effects resulting from the no slip boundary conditions at the sidewalls. The wide channels also provided flexibility in observing transport in the z-direction (Fig. 1) over longer time periods relative to more narrow channel widths.

Flow cells for devices containing rectangular cavities were made using a combined top/middle layer (Fig. 1) of PDMS with a 100 μm deep channel created using methods previously described.²⁸ The width of the channel was 2.5 mm for reasons described above. Inlets and outlets were incorporated into the top/middle layer (Fig. 1) using PDMS tubing (I.D. 0.063 in., O.D. 0.125 in.) (Cole Parmer Instrument Company, Vernon Hills, IL) that was molded into the PDMS during curing. The bottom layer (Fig. 1) of the device was fabricated as described above for the cylindrical cavities, except the cavities were rectangular with sides of 500 μm and depths of 100 μm.

Experimental systems

For experiments involving cylindrical cavities, deionized water was manually loaded into fluid lines (polyetheretherketone tubing, Upchurch Scientific, Oak Harbor, WA). Fluid was pushed through devices containing storage depots using syringe pumps (Kloehn, Las Vegas, NV) at an average velocity of 0.2 mm s⁻¹. A Zeiss ICM-405 inverted epifluorescence microscope (Carl Zeiss Inc., Thornwood, NY) was used to monitor fluorescence in and dissolving from the storage depots. Fluorescence images were captured by an integrating cooled CCD camera (model SBIG ST-7i; Santa Barbara, CA) or stored on VHS tape from images captured in real time using a color video camera (Model 2222-10200000; Cohu, Inc., San Diego, CA), from which temporal data was extracted (Fig. 4).

For experiments involving rectangular cavities, buffer containing RBG was manually loaded into similar fluid lines. Fluid was pushed through devices containing storage depots at an average velocity of 33 mm s⁻¹. A Zeiss IM-35 inverted fluorescence microscope (Carl Zeiss Inc., Thornwood, NY) was used to collect images of the fluorescent dye resorufin that was the product of the conversion of RBG by the enzyme β-gal. Emission signals were captured on a 23-chip cooled color CCD camera (Oncof, Gaithersburg, MD) and also on VHS tape using the same camera, from which temporal data was extracted.

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References


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