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Investigation into the Applicability of the Centrifugal Microfluidics Platform for the Development of Protein−Ligand Binding Assays Incorporating Enhanced Green Fluorescent Protein as a Fluorescent Reporter

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The incorporation of a protein−ligand binding assay into a centrifugal microfluidics platform is described. The platform itself is a disc-shaped polymer substrate, upon which a series of microfluidic channels and reservoirs have been machined. Centrifugal microfluidics platforms require no internal moving parts, and fluid propulsion is achieved solely through rotation of the disc. Fluid flow is controlled by passive valves, the opening of which is dependent on the angular frequency of the rotating platform, the channel dimensions, and the physical properties of the fluid. To evaluate the effectiveness of incorporating a protein-based assay onto the centrifugal microfluidics analytical platform, a class-selective, homogeneous assay for the detection of phenothiazine antidepressants was employed. This class of drugs is known to bind to calmodulin, a calcium binding protein. Specifically, a fusion protein between calmodulin and enhanced green fluorescent protein was utilized. Calmodulin undergoes a conformational change upon binding to phenothiazines that alters the fluorescence properties of the attached fluorescent protein, which can be correlated to the concentration of the drug present. Another important aspect of this work was to study the efficacy of the platform to perform reconstitution assays. To do this, the biological reagent was dried on the platform and rehydrated to carry out the assay. The ability to prealiquot reagents on the platform should enhance its versatility and portability. The integration of protein-based assays in this platform should be useful in the design of analytical systems for high-throughput screening of pharmaceuticals and clinical diagnostics.

Micro total analysis systems (μ-TAS) and microelectromechanical systems have come to the forefront of analytical chemistry in recent years.[1−4] Typically, these devices consist of a series of reservoirs and channels fabricated into silicon, glass, or polymer substrates that can be used to perform specific functions, such as metering, mixing, washing, and calibration, as part of a total analysis.[5,6] These small architectures can be arrayed to create many structures for performing multiple analyses on a single platform. Miniaturization of these devices offers many advantages such as minimized reagent consumption and waste generation, low power and space requirements, ease of portability, decreased analysis times resulting in higher throughput, and decreased total cost.

The demand for μ-TAS devices has led to new advances in microfabrication[7,8] as well as in the understanding of microfluidics.[9,10] By far, fluid manipulation is the most critical factor in the design of these systems. The ability to transport fluids in a controlled and predictable manner is of utmost importance. One of the most popular means of fluid propulsion is electrokinetic pumping, in which the generation of a charged double layer can be used to create a bulk fluid flow upon application of a potential.[6,11,12] This method of pumping is very compatible with materials commonly used in microfabrication; however, electrokinetic flow is strongly dependent on the ionic strength and the pH of the sample matrix.

A promising alternative for fluid transport is centrifugal pumping, in which microdimensioned architectures consisting of channels and reservoirs are machined into a disc-shaped polymer substrate of dimensions similar to a compact disc (CD).[5,13−17] Fluid

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propulsion is achieved through rotation of the disc platform; therefore, fluid flow is dependent on the angular frequency of the rotating platform, the channel dimensions, and the physical properties of the fluid. The centrifugal microfluidics platform uses passive capillary valving to control fluid flow by incorporating burst valves within the channels to create a discontinuity. The fluid becomes "pinned" at this junction. At any time, there are two forces acting on the fluid. The first is primarily due to surface tension, which is dependent on the properties of the fluid and is described in eq 1,

$$\Delta P_s = a(4\gamma/d_H) + b \quad (1)$$

where γ is the surface tension, d_H is the hydrodynamic channel diameter, and a and b are constants that are dependent on the geometry and the wettability of the substrate material, respectively. This is the predominant force at slower rotational speeds. The other is the centrifugal force that is exerted on the fluid as the disc is spun, and is described in eq 2,

$$\Delta P_c = \rho\omega^2\bar{r}\Delta r \quad (2)$$

where ρ is the fluid density, $\bar{r}$ is the average distance of the liquid in the channel from the center of rotation, Δr is the radial extent of the fluid subjected to centrifugal force, and ω is the angular frequency of rotation. When a specific angular frequency is reached, i.e., when ΔP_c exceeds ΔP_s, the fluid will "burst" into the channel.

To demonstrate the versatility of the centrifugal microfluidics platform and to compare its analytical performance to that of more conventional methods,[18] a previously developed assay for the detection of phenothiazine antidepressants was employed. Specifically, we describe the incorporation of a class-selective, homogeneous protein-based binding assay for the detection of phenothiazines within a centrifugal microfluidics platform using fluorescence detection. Similar assays for the detection of this particular class of drugs have been reported, but on the microtiter plate scale.[18,19] These drugs are known to bind to calmodulin (CaM),[20−24] a calcium binding protein, and inhibit it from binding to other signaling proteins.[25] Upon binding to calcium, calmodulin undergoes a conformational change,[26−28] which exposes a hydrophobic pocket in each of its two domains. Calmodulin antagonists can then bind in this hydrophobic region causing yet another conformational change.[29] The latter conformational change can alter the fluorescence properties of an attached fluorescent protein, which can be correlated to the concentration of drug present. In the assay used in our system, enhanced green fluorescent protein (EGFP)[30−32] was attached to the C-terminus of calmodulin through a gene fusion approach. The integration of protein-based assays into a centrifugal microfluidics platform enables the performance of multiple parallel assays on one platform. This platform has the potential for use in high-throughput screening of pharmaceuticals and clinical diagnostics applications. We also demonstrate herein the capability to dehydrate biological reagents within the reservoirs and their subsequent reconstitution[33] in order to make this platform more amenable to field use.

EXPERIMENTAL SECTION

Reagents. Glycine, ampicillin, calcium chloride, tris(hydroxymethyl)aminomethane hydrochloride (Tris HCl), ethylene glycol−bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, anti-FLAG M2 affinity gel, protease inhibitor cocktail, and all other reagents not specified were purchased from Sigma (St. Louis, MO). Trifluoperazine dihydrochloride and propranolol hydrochloride were obtained from Aldrich (Milwaukee, WI). Isopropyl β-D-thiogalactopyranoside (IPTG) was obtained from Gibco BRL (Gaithersburg, MD). Luria Bertani (LB) broth was purchased from Difco (Becton Dickinson, Sparks, MD). The protein assay reagent was procured from Bio-Rad Laboratories (Hercules, CA). Bovine serum albumin was obtained from Pierce (Rockford, IL). The purified rEGFP protein was obtained from Clontech (Palo Alto, CA). The CNC-machined CD was fabricated by Plastifab (Poway, CA). All solutions were prepared using deionized (Milli-Q, Millipore, Bedford, MA) water. All chemicals were reagent grade or better.

Isolation and Purification of the CaM−EGFP Fusion Protein. A glycerol stock of DH5α *Escherichia coli* cells transformed with the plasmid, pSD5100, was used to grow an overnight culture in LB broth containing 100 μg/mL ampicillin at 37 °C. (The preparation of this plasmid is described in detail in ref 18.) Flasks containing 500 mL of media were then inoculated with this overnight culture and were allowed to grow until reaching an optical density of 0.6 at 600 nm. The culture was then placed in a shaker incubator at 30 °C and induced with 1.5 mM IPTG for 6 h. Sonication was performed on the cells to release the cytoplasmically expressed protein. A protease inhibitor cocktail was added

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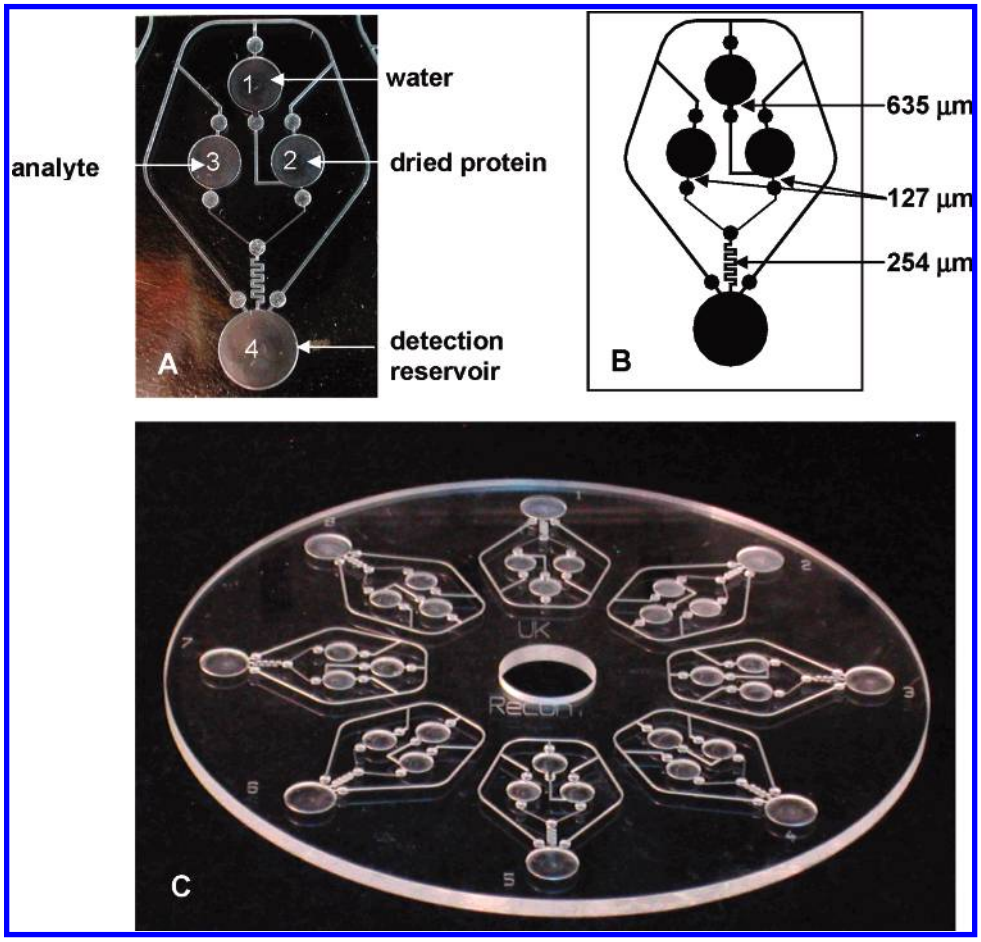

Figure 1. (A) Photograph of the microfluidic architecture used for the protein-based assay, with the reservoirs numbered. The outer channels surrounding the main structure are fabricated into the disc as air channels to prevent back pressure. (B) AutoCAD drawing of the same architecture with the widths of the burst valves and the serpentine mixing structure indicated. (C) Photograph of the entire disc containing eight identical architectures arrayed around a PMMA disc with a diameter of 120 mm. Each individual structure is also numbered.

prior to sonication to prevent proteolysis. Standard molecular biology procedures were followed during the course of this work.[34] After sonication, the crude extract was purified, its purity verified, and its concentration estimated in the manner reported in ref 18.

Centrifugal Microfluidics Platform. The centrifugal microfluidics platform (Figure 1) used in the described experiments was designed specifically for the incorporation of assays in which a reconstitution component was necessary. The design also contained a mixing structure in order to thoroughly mix the reconstituted protein and the analyte. This particular structure was designed using a CAD program, and the design was arrayed around a circular template measuring 120 mm in diameter with a center hole of 15 mm. The resulting architecture was then CNC machined into a poly(methyl methacrylate) (PMMA) substrate of a thickness of 3 mm. All solutions were placed into the reservoirs via pipetting. After filling the reservoirs, each individual structure was covered with optically transparent adhesive tape to enclose the system. The air vents were then punctured with a syringe to reduce the back pressure that is created when the fluids move toward the periphery of the structure upon spinning.

Measurement Setup. Once the substructures were filled, the disc was placed onto the motor hub and secured. The servomotor was run via a controller (PIC−Servo, HdB Electronics, Redwood City, CA) that was interfaced to a PC by a Visual Basic program and powered by a 12-V power supply. This software allowed for control of the acceleration/deceleration rates and the final angular frequency of the rotating platform. After subjecting the disc to a series of accelerations to different angular frequencies to release the fluids from the reservoirs at the desired times, the disc was decelerated to perform the fluorescence measurements. The disk was manually rotated 360° for the measurement of all eight detection reservoirs. A stroboscope with a tachometer (Monarch, Amherst, NH) was used to visualize the fluidics and to measure the angular frequency of the rotating platform.

All fluorescence measurements were performed on a Varian Cary Eclipse (Palo Alto, CA) fluorescence spectrophotometer with a fiber-optic attachment. The fiber optic was positioned perpendicular to the CD platform in the center of the detection reservoir at a distance of ~2 mm and remained stationary throughout the experiment. After deceleration, the CD was manually rotated so that the fiber optic was centered above each detection well. The excitation wavelength was set at 488 nm, and the emission monochromator was set at 508 nm (with a slit width of 5 nm for both) for optimum detection of the fluorescence of EGFP. All fluorescence intensities were background subtracted and are an average four replicates unless otherwise stated.

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RESULTS AND DISCUSSION

The centrifugal microfluidics platform is a relatively new technology that has not been thoroughly investigated. With the ever-increasing interest in microfluidics, this platform offers great potential for the development of many systems. One can avoid problems that arise from the chemical nature of solutions, because the flow within this platform is generic and insensitive to bubble formation and properties such as ionic strength and pH.[13,14] The force that is exerted during spinning of the disc is uniform over the entire platform, which will allow multiple analyses to be performed simultaneously with minimal variability between each structure. To demonstrate the wider applicability of this technology, a well-characterized protein−ligand binding assay[18] for the detection of phenothiazine derivatives was incorporated onto the CD platform. We also demonstrate the ability to dry the biological reagents necessary to perform this assay on the platform itself for easy storage and subsequently reconstitute them as part of the assay.[33]

Microfluidics Platform Performance. As mentioned before, one of the most important aspects of μ-TAS devices is the ability to control the fluidics in a predictable manner. When performing a complete assay in one microfluidic architecture, the gating of the fluids has to be tightly controlled so that releases are in the proper order. Without appropriate releases, adequate reconstitution or thorough mixing cannot be achieved. In the centrifugal microfluidics platform used in these experiments, burst valves were used when a controlled release was required.

In this design, a burst valve of 635 μm in width and 635 μm in depth was incorporated at the base of reservoir 1, which allowed it to release at a moderate angular frequency into reservoir 2 (release 1). The burst frequencies for reservoirs 2 and 3 were designed to be the same (release 2); they both have the same dimensions (127 μm in width and 63.5 μm in depth) and are equidistant from the center of rotation. When the angular frequency becomes sufficiently high that the centrifugal force overcomes the surface tension effect, both reservoirs 2 and 3 release simultaneously; the contents of both meet at the junction and proceed through the serpentine structure that induces mixing. The flow in microchannels of these dimensions is expected to be laminar, but chaotic mixing can occur in this type of structure due to the sharp 90° turns,[10] which cause a tumbling of the fluids. The final destination for the fluids is reservoir 4, where detection is performed using a fiber optic.

The release characteristics of the microfluidics platform were investigated using solutions of colored water to visualize the fluidics, with the understanding that these solutions did not have the exact same physical properties as the protein and drug solutions that would be employed. It was necessary to determine whether the burst valves would perform according to theory and also that there was a discernible separation between the burst frequencies of release 1 and release 2 to ensure that the assay would operate as designed. Samples were placed in reservoirs 1 (blue) and 3 (yellow) and covered as described. The motor control program was set to increase to 1050 rpm with a ramp of 30 rpm/s. A stroboscope was used to enhance our viewing capabilities, and the angular frequency was read from the tachometer. Ten replicate measurements were performed for each of the eight structures on the platform. A graph of these results can be seen in Figure 2. Each of the eight identical structures had reproducible releases in which a good separation between the burst frequencies required for release 1 and release 2 were observed. The releases of valves 2 and 3 were simultaneous within experimental error; therefore, good mixing was achieved as the fluids progressed through the serpentine structure. This was confirmed by the presence of a uniform green solution in reservoir 4.

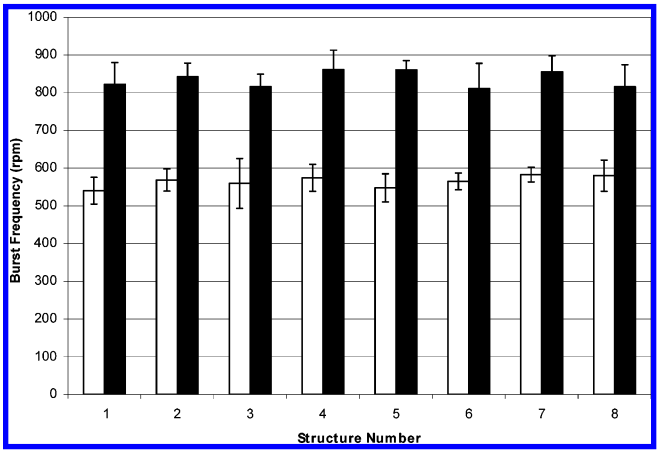


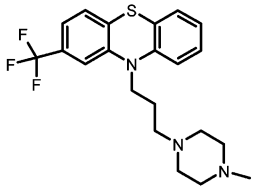
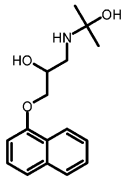
Figure 2. Release profiles of each numbered structure arrayed around the disk platform. The white bars represent the angular frequencies observed upon the release of fluid from reservoir 1. The black bars correspond to the frequencies recorded upon the simultaneous release of reservoirs 2 and 3. Data are the average ± one standard deviation (n = 10).

Fluorescence Studies of CaM−EGFP on the Microfluidics Platform. When dealing with miniaturized platforms and detection systems, it is often difficult to get adequate signal due to path length constraints and small volumes. To determine the optimum concentration of the CaM−EGFP fusion protein required to obtain a reasonable signal using a fiber-optic attachment, a calibration plot was generated. A 25-μL sample of each protein dilution (in Tris buffer) was placed directly into the detection reservoir (reservoir 4 in Figure 1A) on the disk platform. Four samples of each of six different concentrations were used. A graph of fluorescence versus protein concentration yielded a linear plot from 1×10^{-8} to 1×10^{-5} M, in which the detection limit was determined to be 6×10^{-7} M. The calculated reduced χ^2 value was 0.56, indicating a good fit. Given that the assay is based on a decrease in fluorescence of CaM−EGFP, a concentration that is 35-fold higher than the standard deviation of the background, 5.0×10^{-6} M, was chosen to be the final concentration of the fusion protein in the assay. This meant that a solution of 1×10^{-5} M CaM−EGFP had to be placed in reservoir 2, considering that the assay requires mixing of equal volumes from reservoirs 2 and 3.

In this system, the detection of phenothiazines by calmodulin is based on the calcium-dependent conformational change of the protein. It is well documented that calmodulin binds to this particular class of drugs in a predictable manner. Upon binding to phenothiazines, the resultant conformational change perturbs the microenvironment of EGFP, a fluorescent protein, which is directly fused to calmodulin. In doing so, we are taking advantage of this biological recognition element for the detection of phenothiazines.

The same microfluidics design was used for both the aqueous and reconstitution assays. The aqueous assays required use of

Table 1. Comparison of CaM−EGFP Response to Trifluoperazine and Propranolol[a]

Compound	Trifluoperazine	Propranolol
Chemical Structure		
Maximum Fluorescence Quenching % (aqueous)	68	6.2
Detection Limit (aqueous)	7.4×10^{-4} M	N.R.

[a] N.R., no response.

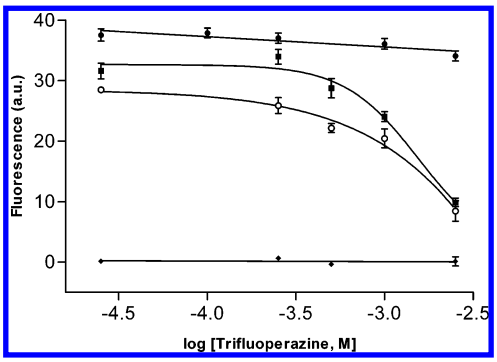


Figure 3. Fluorescence response of the fusion protein (■) aqueous assay and (○) reconstitution assay and rEGFP (●) to varying concentrations of the antidepressant, trifluoperazine. Trifluoperazine (◆) was also tested for any inherent fluorescence response. A final concentration of 5.0×10^{-6} M was used for the fusion protein and 1.35×10^{-6} M for the rEGFP protein. Measurements were blank-subtracted, except the assay involving rEGFP. Data are the average ± one standard deviation of four samples.

only reservoirs 2−4. The protein solution was placed in reservoir 2, while the analyte of interest was placed in reservoir 3. The angular frequency was increased rapidly to a maximum of 1300 rpm. The well-mixed sample then filled reservoir 4, where a 10-min incubation step was used to allow for the binding of the drug and the subsequent conformational change of the protein that is indicative of the presence of phenothiazine derivatives in the sample.

In the reconstitution assay, a 12-μL sample of protein in Tris buffer was placed in reservoir 2 of each of the eight structures on the CD prior to analysis. The platform was then placed in a dark, humidity-controlled environment for 3.5 h to allow the protein to dry. A 12-μL sample of water was then added to reservoir 1. The motor control was set to 675 rpm at a ramp of 200 rpm/s to bring about the release of reservoir 1 into reservoir 2. The spinning of the CD is then stopped for 5 min to allow the protein to reconstitute. Further spinning could cause the premature release of the lower reservoirs due to slow wetting around the valves. As above, reservoir 3 contained the analyte of interest. The angular frequency was then increased rapidly to a maximum of 1300 rpm. The samples were mixed and incubated for 10 min to allow the binding of the drug and the conformational change of the protein to take place.

Both the solution-based assay and the reconstitution assay were performed using the same concentration of the protein and varying concentrations of trifluoperazine. These results can be seen in Figure 3. The fluorescence response decreased at higher concentrations of trifluoperazine. This is due to the pronounced conformational change that occurs upon binding of trifluoperazine to calmodulin. A lower total signal is observed in the reconstitution assay. This is more than likely due to a loss in activity of the protein due to the drying process. A solution-based assay using only the fluorescent protein, rEGFP, with trifluoperazine was also performed. When trifluoperazine was added to the purified rEGFP, there was no appreciable fluorescence change over the concentration range used in the experiment. This was expected, because the recognition element, calmodulin, was not present. Additional measurements were also taken of trifluoperazine in a buffered solution. The drug itself had no inherent fluorescence response.

To demonstrate that the results were indicative of a binding event between calmodulin and the phenothiazine, trifluoperazine, another aromatic compound was also assayed on the CD platform. The compound used was propranolol, a β-adrenergic blocker, which does not bind to calmodulin.[35] The binding of trifluoperazine to calmodulin has a quenching effect on the fluorescence of EGFP at high concentrations; however, there is no change within experimental error in the fluorescence response of EGFP in the presence of propranolol (Table 1). This demonstrates the selectivity of the assay. The detection limit for the trifluoperazine assay was found to be 7.4×10^{-4} M for both the aqueous and the reconstitution assays. This is a strong indicator that the assay's performance is not compromised by the reconstitution of dried reagents on the platform. In comparison to the microtiter plate format,[18] the microfluidics platform performed quite well. A detection limit of 4×10^{-5} M was seen for the microtiter plate assay. A slight increase in detection limits is expected when the assay is scaled down.[19] The reduction in assay volumes is another important advantage in the miniaturization of assays into the centrifugal microfluidics platform. Our system operates with a maximum volume of 25 μL, while an assay performed in a typical 96-well microtiter plate can range from 100 to 300 μL. To demonstrate the feasibility of our approach, we used reservoirs

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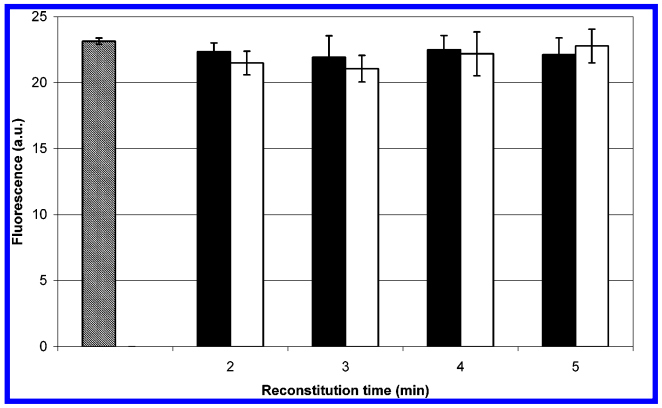

Figure 4. Optimization of reconstitution times. The gray bar represents a solution-based dilution of the protein, i.e., maximum signal. The black bars (■) are the fluorescence counts obtained after reconstitution of the same protein dilution that had been dried for 3.5 h. The white bars (□) represent the counts acquired after the protein was dried overnight (~16 h). Data are the average ± one standard deviation of four samples.

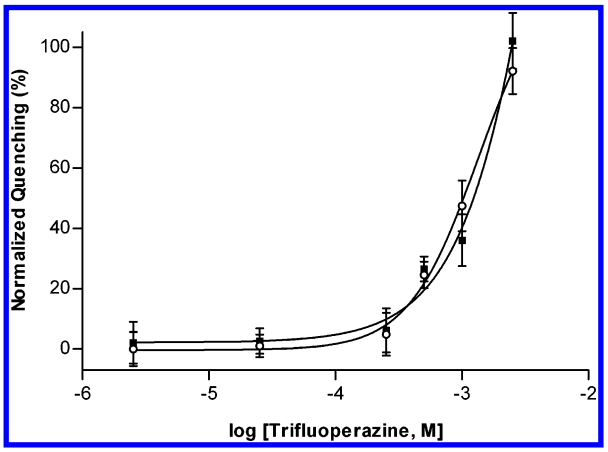

Figure 5. Dose−response curves for trifluoperazine after performing the reconstitution assay. The dried protein was reconstituted for 5 min and was then incubated with the drug for 5 (■) and 10 min (○). Data are the average ± one standard deviation of four replicate measurements.

of microliter volume size (~12 μL). However, we have employed microfabrication methods that are scalable to the nanoliter volume size. Thus, the described methods are fully scalable to volumes below those of 384-well microtiter plates.

Optimization of the Microfluidics Platform. The assays that have been described to this point have analysis times between 10 and 15 min. To lower the analysis time, experiments were performed on the reduction of both the reconstitution time of the CaM−EGFP reagent and the incubation time with the drug in reservoir 4. Figure 4 depicts the results obtained in the attempt to lower the reconstitution time. Included in this graph is a comparison between a 3.5-h drying time and an overnight drying interval. The results were compared to a protein solution of the same concentration. It appears that a 2-min reconstitution time would be sufficient to maintain the assay's performance. All of the results are within 10% of the actual expected fluorescence values.

A comparison was also made in order to decrease the time needed for incubation between CaM−EGFP and the drug in reservoir 4 before measurement. Figure 5 contains normalized fluorescence response curves that were generated as a result of performing the reconstitution assay with 5- and 10-min incubations. The graphs are almost identical; therefore, it would be possible to perform the analysis with only a 5-min incubation step. This is a significant improvement over the 15 min required to perform the same assay in a microtiter plate regime.[18] This is due to the smaller volumes, in which diffusion and the binding of the drug to calmodulin take less time. After performing optimization experiments, the analysis time was decreased by at least 50% to between 5 and 7 min, allowing for faster results while still maintaining accuracy. To demonstrate the feasibility of our approach, we used reservoirs of microliter volume size (~12 μL).

CONCLUSIONS

It has been demonstrated that the centrifugal microfluidics platform employed in this study can be useful in the performance of homogeneous, protein-based assays using fluorescence detection. The ability to perform such assays establishes the platform's potential usefulness as a tool for performing multiple parallel analyses. Within this framework, numerous assays can be performed for different analytes by using identical structures within a single CD or a standard calibration can be performed alongside a set of analyses. Further miniaturization and changes in the structural design of the microfluidics platform is possible and would allow an even greater number of parallel assays to be performed. We have also successfully established the ability to dry biological reagents and later reconstitute them on the microfluidics platform without inhibiting the performance of the assay. This enhances the long-term storage capabilities of this system. In summary, this paper integrates a binding assay involving reconstitutable reagents within a microfluidic platform. This is, to the best of our knowledge, the first report that combines all these features. The only other report that uses reconstitutable reagents was just published[36] and does so by continuous dissolution from a pit on the side of a microfluidic channel using a conventional pumping method. The advantage of our method is that a valve is used to confine reagent reconstitution to a reservoir. This passive valve is closed as long as the rpm of the microcentrifuge is set below a set value. This allows for full reconstitution of the reagent. Spinning at a higher rpm releases the reagent through the microfluidic network to complete the analysis.

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