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SHORT COMMUNICATION

Refolding of β-galactosidase: microfluidic device for reagent metering and mixing and quantification of refolding yield

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Abstract We have developed a device that uses microfluidic valves and pumps to meter reagents for subsequent mixing with application to refolding of the protein β-galactosidase. The microfluidic approach offers the potential advantages of automation, cost-effectiveness, compatibility with optical detection, and reduction in sample volumes as opposed to conventional techniques of hand-pipetting or using robotic systems. The device is a multi-layered poly(dimethylsiloxane) on glass device with automated controls for reagent aliquoting and mixing. Refolding experiments have been performed off-chip using existing protocols on the protein β-galactosidase and the refolding yield has been quantified on-chip using fluorescein di-β-D-galactopyranoside, a caged-fluorescent molecule. This work provides the potential to reduce the cost of drug discovery and realization of protein pharmaceuticals.

Keywords Protein refolding · β-Galactosidase · Combinatorial · Microfluidic

1 Introduction

Protein refolding has been a bottleneck in the production of biopharmaceuticals on a large scale. Synthesis of recombinant proteins in the biopharmaceutical industry uses bacterial systems like *Escherichia coli* as hosts. To overexpress the production of recombinant proteins, promoters are used to induce genetically modified bacteria. This overexpression leads to the formation of misfolded protein aggregates called inclusion bodies (Williams et al. 1982). The process of refolding involves the conversion of these non-functional protein aggregates to their functional native state. However, in most cases, the solution conditions to properly refold proteins are not known a priori. Determination of these solution conditions is a highly empirical process.

The process of finding the reagents and buffers to properly refold proteins has been conventionally done by hand-pipetting, which is a highly time and labor-intensive process, or using robotic systems, which are very expensive. In either case, proteins are mixed combinatorially with various reagents on a 96-well plate, thus requiring expensive proteins and reagents on the order of several milliliters. The refolding yield is then quantified using various techniques like immuno affinity (Teale and Benjamin 1976a, b) or spectroscopic methods (Pelton and McLean 2000).

The microfluidic approach has many potential advantages compared to conventional techniques. The sample volumes used in microfluidic devices are on the order of nanoliters as opposed to milliliters used in the conventional methods, resulting in a million-fold volume reduction. Additionally, no expensive robotic control is involved for automation of fluid handling. Both these factors contribute to the cost-effectiveness of the microfluidic device.

Kinetic measurements of protein conformation during unfolding and refolding are important in validating models for the folding process. Different methods are employed on microfluidic platforms to study the intermediate protein states like fluorescent dyes that bind to exposed hydrophobic regions of partially folded proteins (Kerby et al. 2006) or techniques like small-angle X-ray scattering (Pollack et al. 2001) and terahertz spectroscopy (George

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et al. 2008). Our device is compatible with on-chip optical detection, which gives the potential for real-time monitoring of these kinetic intermediates using the various techniques mentioned above.

The upcoming sections are organized as follows: first, details of refolding of the protein β-galactosidase are discussed. Next, materials and methods to fabricate the device and implement the refolding protocol are discussed. The next section deals with device design to achieve refolding on-chip. The last few sections present the results, discussion and conclusion.

2 β-Galactosidase refolding

Refolding experiments are performed on the protein β-galactosidase, which is an essential enzyme in the human body that catalyzes the hydrolysis of β-galactosides like lactose into monosaccharides (Wallenfels and Weil 1972). Commercial preparations of the enzyme are used in the preparation of lactose-free products and tablets to cater to the large lactose intolerant population. Apart from its biological activity, the enzyme has gained importance in molecular biology as a reporter protein (Jacob and Monod 1961; Beckwith 1967). β-galactosidase is encoded by the *lacZ* gene of the lac operon in *E. coli*. Activity of a promoter that is fused to the *lacZ* gene can thus be detected by measuring the levels of β-galactosidase (Jacob and Monod 1961; Beckwith 1967).

The structural and enzymatic properties of the enzyme have been well characterized (Jacob and Monod 1961; Karlsson et al. 1964; Craven et al. 1965; Marchesi et al. 1969; Huber et al. 1976; Huber et al. 1984; Jacobson et al. 1994). It is one of the largest tetrameric enzymes known at present, with a total of 1023 amino acids (Fowler and Zabin 1978; Kalnins et al. 1983). Early studies on the protein showed that it can be denatured in the presence of high concentrations of urea (Shifrin and Steers 1967; Marchesi et al. 1969; Ullmann and Monod 1969; Nichtl et al. 1998) while the folding pathway of the protein was studied by Nichtl et al. (1998).

The denaturing protocol for the protein involves solubilizing in a buffer solution containing 8 M urea (Nichtl et al. 1998). The solubilized protein along with the denaturants is then diluted in a renaturing medium which mainly contains low concentration denaturing agent (1.4 M urea) and Mg^{2+} ions that aid refolding (Nichtl et al. 1998).

In this paper, we show the potential of our microfluidic device in automating fluid handling for performing protein refolding trials. On-chip refolding yield measurements are performed using a fluorometric assay on the protein β-galactosidase.

3 Materials and methods

3.1 Reagents

Urea, dithioerythritol (DTE), sodium phosphate, 1,1,2,2 tetrahydro-perfluorooctyltrichlorosilane, β-galactosidase from *E. coli* and dimethyl sulfoxide (DMSO) were from Sigma Aldrich (St. Louis, MO). Ethylene diamine tetra-acetic acid (EDTA) and magnesium chloride were from EMD chemicals (Gibbstown, NJ). Fluorescein di-β-D-galactopyranoside (FDG) was from Invitrogen (Carlsbad, CA).

3.2 Device fabrication

The microfluidic device was fabricated using replica molding of poly(dimethylsiloxane) on microfabricated molds (Unger et al. 2000). Device geometries were defined using L-Edit CAD software (Tanner Research). Two mask patterns were created using a GCA/Mann 3600F Optical Pattern Generator—one for the 'flow channels', the channels through which the various reagents flow and the other for the 'control channels', the channels through which pressurized air flows. The silicon wafer used as a master for the flow channels was coated with a 9-μm thick layer of SPR 220-7.0 photoresist and the wafer used for the control channels was coated with a 15-μm thick layer of SU-8 2010 photoresist. The wafers were then exposed using the corresponding masks using an EV 620 alignment tool. The SPR resist was developed using a 300MIF resist developer and the channels were rounded by reflowing at 120°C for 2 min. The SU-8 resist was developed using SU-8 developer and rinsed with isopropyl alcohol and deionized water. Both the masters were surface treated by placing 30 μl of 1,1,2,2 tetrahydro-perfluorooctyltrichlorosilane in a vial along with the masters in vacuum for 2 h (Duffy et al. 1998).

The PDMS layer containing the flow channels was about 5 mm thick and was prepared by mixing the two-part silicone elastomer (Dow Corning Sylgard® 184) in 5:1 ratio and curing it over the flow master. The layer containing the control channels was about 17 μm thick and was prepared by mixing the elastomer in 20:1 ratio and spin coating the control master at 1,750 rpm for 60 s. The two PDMS layers were activated in the plasma cleaner and bonded together (Duffy et al. 1998). The two layered device was then bonded to a glass slide by a similar procedure. Tygon tubes were attached to the reservoirs of the control and flow channels. The control lines were connected to eight-channel manifolds that were controlled through a BOB3 breakout controller board (Fluidigm, San Francisco, CA). The opening/closing of valves and frequency of actuation of the pumps were controlled using an NI-DAQ card

(NI 6533, National Instruments) connected to the breakout board. The control interface was created using LabView program that allows for automation of the control process.

3.3 Device preparation

In order to prevent the adhesion of proteins and other particles to the PDMS channels, the channel walls were coated with a layer of bovine serum albumin (BSA) which preferentially adheres to the channels. The channels were thoroughly purged with deionized water before performing multiple refolding trials. Implementing the refolding protocol on-chip requires long incubation times which may result in the evaporation of the reagents through PDMS. This can be prevented by placing the device in a water bath during long incubation hours.

3.4 Denaturing and refolding protocols

The denaturing and refolding protocols listed here were performed off-chip using standard pipetting techniques. β-Galactosidase was denatured by incubating 35 μg/ml of the protein in 8 M urea, 1 mM ethylene diamine tetraacetic acid (EDTA), 10 mM dithioerythritol (DTE), 0.1 M sodium phosphate (pH 7.5) at 25°C for 2 h. Refolding experiments were carried out by diluting the solubilized protein along with the denaturants 1:12 in a refolding medium containing 0.1 M sodium phosphate, 1 mM magnesium chloride, 5 mM DTE, 1.4 M urea (pH 7.5). The protein was incubated in the refolding medium at 10°C for 30 min and further incubated at 20°C for 12 h (Nichtl et al. 1998).

3.5 Activity assay

The activity of β-galactosidase was determined by a fluorometric assay using fluorescein di-β-D-galactopyranoside (FDG) as the substrate (Rotman et al. 1963; Nolan et al. 1988). Nonfluorescent FDG was sequentially hydrolyzed by the enzyme, first to fluorescein monogalactosidase (FMG) and then to fluorescein, which is highly fluorescent. A 20 mM stock solution of FDG was prepared by dissolving 5 mg in 8:1:1 H_2O/DMSO/ethanol solution. The assay was performed off-chip in a well plate by mixing equal volumes of refolded protein and FDG diluted to 0.2 mM solution, allowing 2 h of incubation at room temperature.

3.6 Fluorescence measurements

The denatured and refolded protein samples were mixed with the diluted FDG solution off-chip and were allowed to incubate for 2 h. The incubated mixtures were then introduced individually into the microchannels of the device. The emitted fluorescence was observed in the microchannel region using a Nikon TE2000U inverted fluorescence microscope with fixed excitation. Images were obtained using a Q-Imaging Retiga camera and Phylum software. Image analysis was carried out in MATLAB.

3.7 Image processing

The volume injection rate of fluid in a flow channel was determined by monitoring the interface between water and dye and estimating the elapsed time for the dye–water interface to move a given distance using image processing tools in MATLAB. The mixing time of reagents in the annular mixer was estimated by visual inspection by filling about half the annulus with water and the other half with dye and actuating the peristaltic pump on the annulus.

4 Device design

The microfluidic device was designed to enable automated fluidic handling of reagents. This requires precise control over the choice and the amount of the various reagents which was achieved using a design consisting of two layers of poly(dimethylsiloxane) (PDMS) on glass (Unger et al. 2000). PDMS is a popular elastomeric material that has been used for a number of microfluidic applications (Thorsen et al. 2002; Lee et al. 2003; Wang et al. 2004). A schematic of the channel layout is shown in Fig. 1. The protein solution and the various reagents flow through the microchannels in the top thick PDMS layer. The channels in this layer, called the 'flow channels', are 90 μm wide.

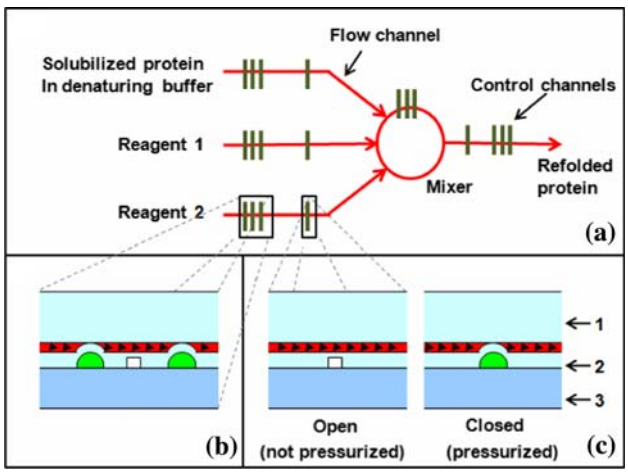

Fig. 1 **a** Schematic of the channel layout showing the flow channels and control channels; **b** peristaltic pump action in the device; **c** valve action in the device

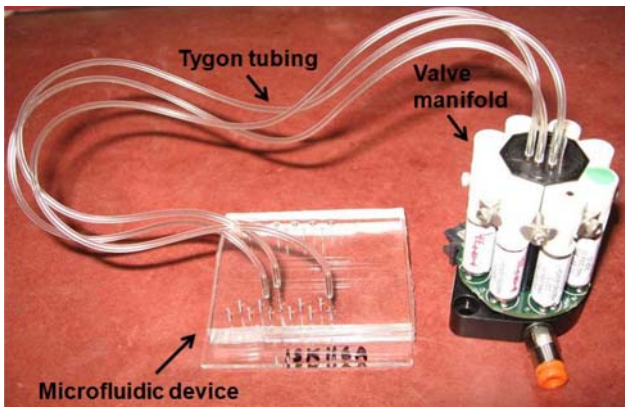

Fig. 2 Microfluidic device integrated to an eight-valve manifold through tygon tubing. The size of the glass slide to which the PDMS layers are bonded is 2″ × 3″

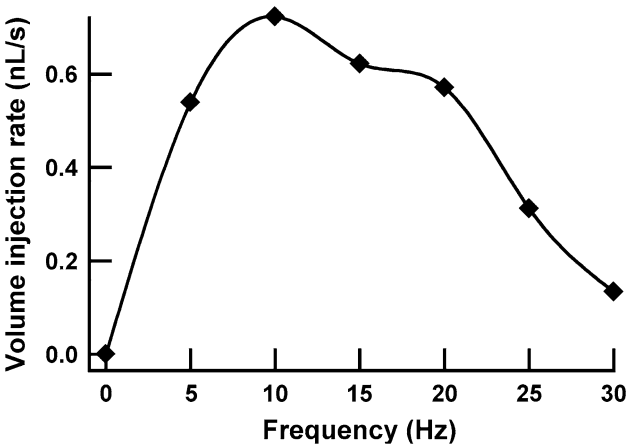

Fig. 3 Volume injection rate in a flow channel plotted against the frequency of actuation of the control channels

Pressurized air was passed through the microchannels in the thin PDMS layer to control the flow of reagents in the flow layer. The channels in this thin layer, which is sandwiched between the top thick PDMS layer and the glass slide, are called 'control channels' and these channels are 120 μm wide. Each flow channel was equipped with a valve and pump to control the choice and amount of the reagents flowing through it. The chosen reagents were then mixed with the protein solution in the annular mixer (Chou et al. 2001) which has a radius of 1,270 μm. The output was the protein refolded to different degrees depending on the solution conditions. The process was automated using a proprietary circuit and a LabView program. A picture of the device connected to a valve manifold is shown in Fig. 2. The degree of refolding was then quantified using a fluorometric assay (Rotman et al. 1963; Nolan et al. 1988).

The preliminary design of the device contains three input channels for conducting refolding trials. Subsequent versions can be expanded by incorporating multiple input channels for implementing complex combinatorial refolding protocols.

5 Results

Results described in this section are as follows: (1) the device was characterized by determining the optimum pumping frequency and mixing time of reagents in the annulus; (2) the refolding protocol and the fluorometric assay were optimized off-chip for best on-chip signal intensity; (3) the fluorescence intensity was then calibrated as a function of β-galactosidase concentration in a PDMS microchannel; (4) finally, the active protein content in denatured and refolded protein samples was quantified on-chip using the obtained calibration curve.

5.1 Determination of optimum pumping frequency

Fluid actuation was characterized to facilitate reagent metering. The flow rate of the fluid in the channel depends on the frequency of actuation of the peristaltic pump on the flow channel (Unger et al. 2000). The peristaltic actuation of the control channels creates a positive displacement which results in fluid flow. The volume injection rate was calibrated against the frequency of actuation of the control channels as shown in Fig. 3. The volume injection rate was determined using techniques described in Sect. 3.7. The flow rate is maximum at a pumping frequency of about 10 Hz, beyond which the valve opening/closing is incomplete.

5.2 Reagent mixing in the annulus

The mixing time of reagents in the annulus was estimated by performing dye-water mixing experiments. Water and dye were introduced through the input channels into the mixing ring and the valves on all the channels connected to the ring were closed. The pump on the ring was then actuated which resulted in the mixing of the water and dye solutions. Snapshots of the device during mixing are shown in Fig. 4. The mixing time was estimated as 45 s using techniques described in Sect. 3.7. The pump on the annulus is operated at 10 Hz actuation frequency.

5.3 Determination of optimum FDG incubation time

As described in Sect. 3.5, nonfluorescent FDG is used as the substrate for measuring β-galactosidase activity. β-Galactosidase sequentially hydrolyzes FDG first to FMG (fluorescein monogalactoside) which is not fluorescent and then to fluorescein which is highly fluorescent. The turnover rate for the hydrolysis of FDG to FMG is relatively slow

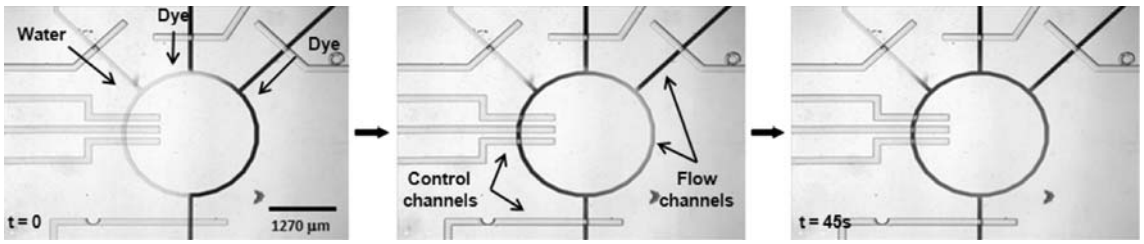

Fig. 4 Snapshots of the device at different times during mixing. Complete mixing is accomplished in about 45 s. The flow rate of the fluid in the mixing ring upon actuation of the peristaltic pump is 1.07 nl/s

(1.9 µmol min^{-1} mg^{-1}) compared to the rate of conversion of FMG to fluorescein (22.7 µmol min^{-1} mg^{-1}) (Huang 1991). This multistep hydrolysis results in a delay in the production of fluorescence. Hence for obtaining sensitive measurements, very short incubation times, typically less than 10 min, should be avoided.

The fluorometric activity assay for quantifying the activity of β-galactosidase was performed with different FDG incubation times ranging from 0 to 270 min. The incubation was performed off-chip at room temperature. Active β-galactosidase at a very low concentration of 0.02 units/ml was used for these trials. A plot of the fluorescence signal intensity as a function of the incubation time is shown in Fig. 5. Increasing the reaction time results in an overall increase in signal. However, the curve describing the increasing trend in fluorescence begins to flatten at long incubation times (greater than 90 min.). All subsequent activity measurements were performed with an FDG incubation time of 120 min, based on the FDG–β-galactosidase assay protocol in Huang (1991).

5.4 Determination of optimum dilution ratio

The refolding protocol was optimized off-chip by performing experiments with different dilutions of the solubilized protein in the renaturing buffer. The protein was denatured following the same protocol described in Sect. 3. During refolding, the solubilized protein was diluted in different ratios in the renaturing buffer: 8, 10, 12, 18, and 36. FDG was added to these samples and incubated for 120 min. The fluorescence signal intensity was plotted as a function of dilution as shown in Fig. 6. A dilution ratio of 12 results in the maximum fluorescence signal and this ratio was used in all subsequent refolding experiments.

5.5 Calibration of fluorescence intensity in a PDMS microchannel

In order to quantify the refolding yield of β-galactosidase on-chip, the fluorescence intensity of the FDG–protein mixture was calibrated in the PDMS microchannel as a function of β-galactosidase concentration as shown in Fig. 7. Active β-galactosidase was used for these measurements with concentrations corresponding to 2.5, 5, 10, 20, and 40 units/ml. The fluorescence intensity was calculated by processing fluorescent micrographs of the channel. The flattening of the curve observed at high protein concentrations is due to pixel saturation.

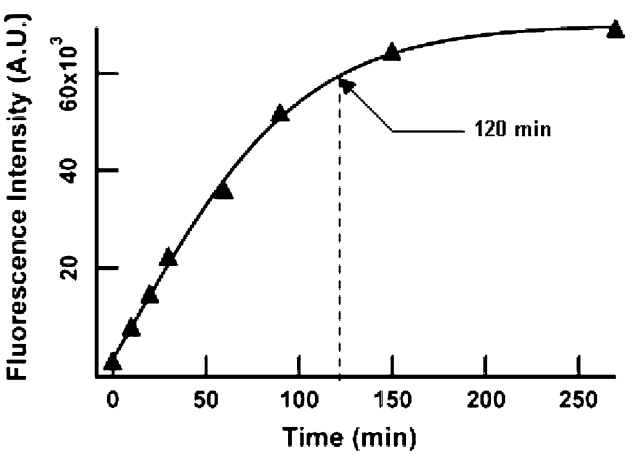

Fig. 5 Fluorescence signal as a function of FDG–β galactosidase incubation time. The line is a sigmoidal curve fit to the data

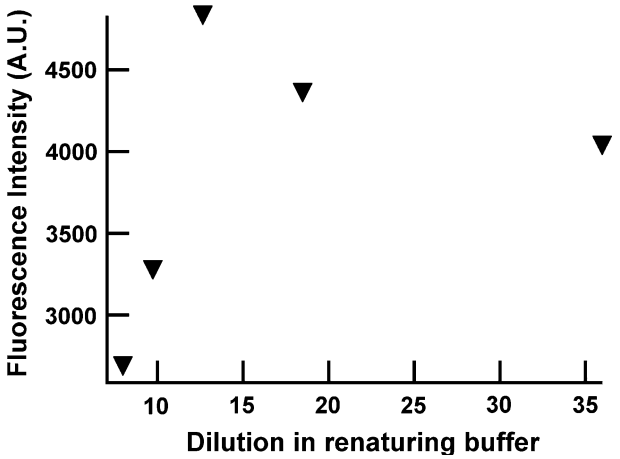

Fig. 6 Fluorescence signal intensity plotted as a function of dilution of the solubilized protein in the refolding buffer

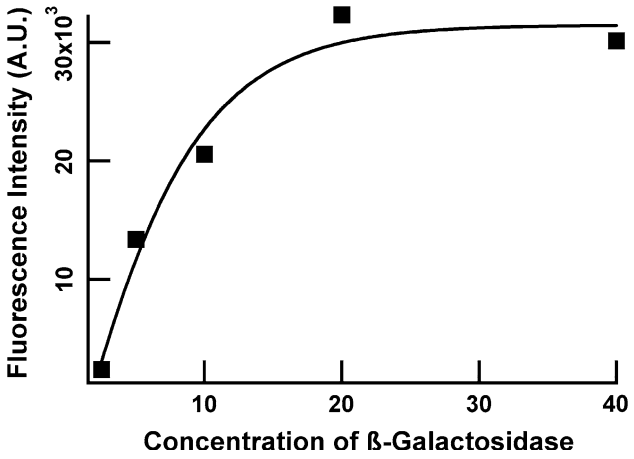

Fig. 7 Calibration curve of β-galactosidase concentration versus fluorescence intensity in a PDMS microchannel. The intensity is calculated by processing fluorescent micrographs of the channel

5.6 Quantifying active protein content in denatured and refolded samples on-chip

Multiple denaturing and refolding experiments were performed with β-galactosidase as per the optimized protocols described in Sect. 3. The refolding yield was determined using the fluorometric assay by quantifying the fluorescence intensity of denatured and refolded samples in the microchannel using image processing techniques. The micrographs of the device filled with denatured and refolded protein samples were used in conjunction with the calibration curve to estimate the refolding yield as 36.84 ± 20.96% as shown in the bar graph in Fig. 8.

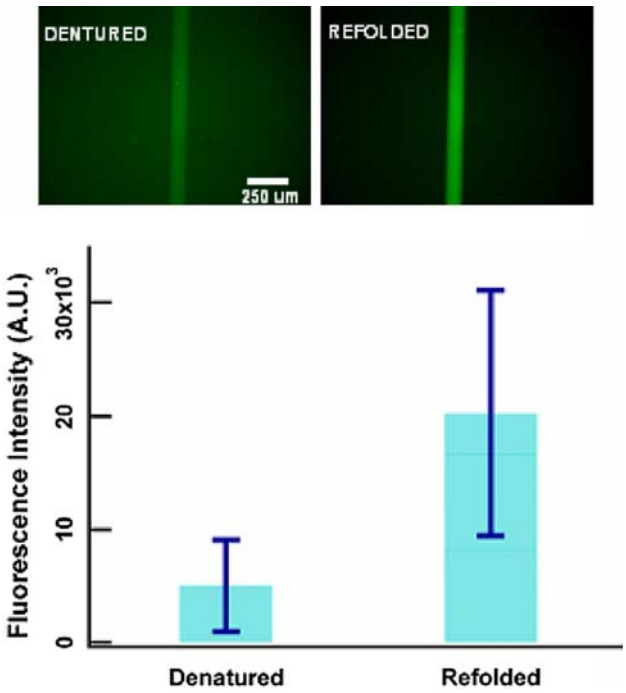

Fig. 8 Micrograph of device filled with denatured and refolded β-galactosidase samples. The protein samples are incubated with FDG off-chip and introduced into the microchannels to quantify the yield. The bar graph shows the fluorescent intensities of the denatured and refolded samples ($n = 3$)

6 Discussion

The refolding yields obtained in these trials are on the order of 36.84 ± 20.96%, comparable to the yields achieved in the existing literature which were on the order of 35–40% (Nichtl et al. 1998). While these yields are still relatively low compared to the percentage refolding that has been achieved for other proteins such as lysozyme (Hevehan and De Bernardez Clark 1997), these results demonstrate that on-chip reagent mixing characterization can be brought to bear on refolding problems.

The main challenge in refolding proteins is the competing kinetics between aggregation and refolding (Zettlmeissl et al. 1979). The aggregated states correspond to local minima in the protein energy landscape (Smeller et al. 2006). These metastable states encountered in the folding pathways result in the formation of inclusion bodies. The most common technique to overcome aggregation is to use very dilute solutions of protein during refolding. However, dilute solutions are not cost-effective. Hence recent refolding trials have resorted to the use of small concentrations of denaturants and other reagents that aid refolding by minimizing aggregation. These synthetic reagents which comprise different types of surfactants, salts and sugars that aid in refolding are called artificial chaperones (Hevehan and De Bernardez Clark 1997). By combinatorially choosing these artificial chaperones using our microfluidic device, the refolding yield can be improved.

Another technique to improve the refolding yield is to add molecular chaperones like GroEL to the renaturing buffer, that aid in folding by encapsulating individual proteins, thereby preventing aggregation (Ayling and Beneyx 1996). It has been shown that the presence of GroEL results in a twofold increase of the in vitro refolding yield of β-galactosidase (Ayling and Beneyx 1996).

As mentioned earlier, our microfluidic approach provides a cost-effective way to implement refolding protocols because the volumes of reagents used in the trials are on the order of nanoliters and automation does not require the use of expensive robotic systems. Since the device is optically clear, it is compatible with on-chip detection of protein intermediates. The process of scaling up to implement complex combinatorial protocols involves the fabrication of multiple refolding mixers on a single chip to parallelize the reactions. It has been shown that microfluidic large-scale integration using multiplexors allows control over a

large network of fluidic channels with minimal number of inputs (Thorsen et al. 2002).

7 Conclusion

We have shown the potential of our microfluidic approach in automating fluid handling and have evaluated protein refolding protocols with on-chip measurements. We have demonstrated the capability of our device in controlling reagent metering and mixing by performing experiments with dye–water solutions and mixing them in an annular mixer in an automated fashion. We have optimized a refolding protocol and fluorometric assay off-chip for best on-chip signal intensity, and shown that denatured and refolded samples of the protein β-galactosidase can be quantified on-chip using a fluorescence based assay.

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