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ORIGINAL PAPER

Refolding of a membrane protein in a microfluidics reactor

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Abstract Membrane protein production for structural studies is often hindered by the formation of non-specific aggregates from which the protein has to be denatured and then refolded to a functional state. We developed a new approach, which uses microfluidics channels, to refold protein correctly in quantities sufficient for structural studies. Green fluorescent protein (GFP), a soluble protein, and bacteriorhodopsin (BR), a transmembrane protein, were used to demonstrate the efficiency of the process. Urea-denatured GFP refolded as the urea diffused away from the protein, forming in the channel a uniform fluorescent band when observed by confocal microscopy. Sodium dodecyl sulphate-denatured BR refolded within the channel on mixing with detergent–lipid mixed micelles. The refolding, monitored by absorbance spectroscopy, was found to be flow rate dependent. This potential of microfluidic reactors for screening protein-folding conditions and producing protein would be particularly amenable for high-throughput applications required in structural genomics.

Keywords Green fluorescent protein · Bacteriorhodopsin · Microfluidics reactor · Membrane protein refolding · Structural genomics

Abbreviations

BR	Bacteriorhodopsin
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulphonate
DMPC	Dimyristoyl-phosphatidylcholine
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetate
GFP	Green fluorescent protein
GST	Glutathione S-transferase
SDS	Sodium dodecyl sulphate
UV	Ultraviolet

Introduction

The structural study of membrane proteins constitutes an important and difficult area of cellular and molecular biophysics — important, because membrane proteins represent 30–40% of the proteins encoded in the human genome and 70% of current drug targets — difficult, because membrane protein purification, solubilisation by detergents or lipids, reassembly and crystallisation remain massive challenges in biochemistry. Currently,

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the protein structure data bank contains over 30,000 structures, of which fewer than 200 are for membrane proteins (Raman et al. 2006). Recently, the application of high-throughput methods to purification, crystallisation and structural determination has accelerated significantly the rate of structural determination for soluble proteins. Because of the inherent difficulties mentioned above, structural genomic projects of membrane proteins lag far behind. A substantial effort is invested in the development of reproducible methods for crystal growth, not only from detergent solution, but also from more physiological lipid environments. This is illustrated by the remarkable work on the archaeal rhodopsins, including the first complex of two membrane proteins: sensory rhodopsin II and transducer II that mediate phototaxis in *Natronobacterium pharaonis* (Gordeliy et al. 2002). In parallel with crystallisation efforts for X-ray studies, membrane protein as well as relevant lipid structures are being investigated by a wide range of biophysical methods including X-ray and neutron solution scattering (Gabel et al. 2002), solid-state NMR (Watts 2005), electron microscopy (Subramaniam et al. 2002), atomic force microscopy (Engel and Muller 2000) and Fourier transform infrared spectroscopy (Nyquist et al. 2004). A biochemically well-purified and well-characterised preparation of the membrane protein, however, is an essential prerequisite for all these studies as well as, in the case of diffraction methods and NMR, milligram amounts of sample material.

Obtaining the correctly folded native state continues to pose an important technical challenge during the production of recombinant proteins. *Escherichia coli* is the oldest of the expression systems used for the production of recombinant proteins and it remains very popular as it is well understood, easy to use, relatively cheap, quick to use and offers the possibility of isotope labelling. A limitation of *E. coli* recombinant protein production is that most eukaryotic proteins form insoluble aggregates or inclusion bodies. In structural genomics efforts targeted at non-membrane proteins from the *Methanobacterium thermoautotrophicum* genome, approximately 40% of expressed proteins did not form soluble protein but accumulated as an insoluble aggregate (Christendat et al. 2000). Similarly for human gene constructs expressed in *E. coli*, it is estimated that only 15–20% form soluble protein, 20–40% form inclusion bodies with the remainder being poorly expressed or degraded (Tresaugues et al. 2004). Strong denaturants (like urea and guanidine-HCl) or detergents [like sodium dodecyl sulphate (SDS)] are required to solubilise the protein in inclusion bodies; most of its structure is lost during this process; and the protein needs to be folded into its native configuration. This procedure has been successful not only for soluble proteins, but also for several integral membrane proteins (Booth 2003).

To meet the requirements of structural genomics projects, a folding technique has to be broadly applicable to a wide range of proteins. Folding of proteins in the laboratory is not a straightforward process, often requiring extensive trial-and-error experimentation (Stevens 2000), making essential a high-throughput screening procedure. Automated refolding by rapid dilution has been attempted with some success using a multiwell plate format with chemical panels generated using a fractional factorial approach (Armstrong et al. 1999; Scheich et al. 2004). However, attempts to scale up the process to produce sufficient material for structural studies have often been unsuccessful. An alternative approach is to scale the process down to a millilitre-sized reactor, where folding takes place within a capillary or microfluidic channel. Previous microfluidic devices developed for proteins were designed to study the initial refolding events (μs–ms) by *rapidly mixing* a denatured protein solution and a refolding buffer through either turbulent flow (Akiyama et al. 2000; Shastry et al. 1998; Sudarsan and Ugaz 2006) or through diffusion (Hertzog et al. 2004); neither devices were, however, optimised for continuous protein production nor described for refolding integral membrane proteins.

The basis of the microfluidics device detailed in this paper is to produce protein by *slowly mixing* through diffusion a denatured protein solution and a refolding buffer flowing parallel to each other (in a laminar manner) through a capillary. This approach can be moreover automated and offers a generic method to screen refolding conditions and subsequently refold protein in quantities sufficient for structural studies. Green fluorescent protein (GFP), a soluble protein, and bacteriorhodopsin (BR), a transmembrane protein, were used to develop and illustrate folding within microfluidic channels. Both proteins have spectral properties that make their effective refolding easy to demonstrate and follow.

Green fluorescent protein, from the jellyfish *Aequorea victoria*, is a single domain soluble protein of 238 amino acids, which generates its own fluorophore in an autocatalytic fashion. Upon excitation at 395 or 475 nm, wild-type GFP emits green light with an emission maximum around 508 nm (Morise et al. 1974). The protein adopts an 11-stranded β-barrel structure. The fluorophore spans the inner portion of the cylinder, isolated from the bulk solvent (Cody et al. 1993). Since it only forms after protein folding, its fluorescence can

be monitored as a criterion for GFP folding correctly. Indeed, GFP, denatured either by acid (Enoki et al. 2004), urea (Reid and Flynn 1997) or guanidine (Fukuda et al. 2000), has been successfully refolded by dilution into appropriate refolding buffers. The fluorescent characteristics of GFP have moreover been used as indicators for correct refolding of a target protein, engineered with a fusion GFP tag (Waldo et al. 1999).

Bacteriorhodopsin is an integral membrane protein that acts as a light-driven proton pump and is the sole protein component of the purple membrane of the archeabacterium, *Halobacterium salinarium*. BR is a single polypeptide that folds to a seven-helix transmembrane structure and binds a retinal chromophore (Haupts et al. 1999). Time-resolved changes in the retinal absorption band has enabled the kinetics of BR folding to be studied. The absorption spectrum of the bound chromophore is significantly different from that of free retinal (absorbance peak at 550–560 nm, instead of 380 nm) and can serve as an easily measurable indicator for the active protein. BR has been refolded in vitro in a variety of detergent and lipid systems (reviewed in Seddon et al. 2004). As with other transmembrane proteins (Baneres et al. 2003), the recovery yields were strongly affected by the concentrations and types of lipids and detergents used in the refolding process.

Experimental

Microfluidic device fabrication

Glass wafers (Soham Scientific, Ely, UK) were cleaned using a piranha solution [3:1 mixture of concentrated sulphuric acid (H_2SO_4) with hydrogen peroxide (H_2O_2)]. The wafers were then blow-dried using a clean nitrogen supply after being rinsed several times in Milli-Q water to remove any residual impurities. A microchannel design, as detailed in Fig. 1, was fabricated on the cleaned wafers using standard photolithographic techniques (Geppert 1996; Jeong et al. 1994; Levenson 1995; Okazaki 1991). A technical drawing software package was used to create a scaled black-and-white artwork of the microchannel design, which was then transferred onto a high-resolution photographic negative using standard photography techniques. Thin films of approximately 250 μm Microchem SU82100 photoresist (Microchem, Newton, MA, USA) were coated over the glass wafers using a Karl Suss Delta 10 spin coater (Karl Suss, Germany) and were pre-baked at 65°C for 12 min and 95°C for 60 min. The photographic negative was used to project an ultraviolet (UV) light source (340 nm) from a mask aligner (Karl Suss MJB3) onto the substrates coated with the film of photoresist. The exposed films of photoresist were then post-baked at 65°C for 1 min and 95°C for 21 min before being developed using an EC Solvent developer solution (Microchem, Newton, MA, USA) to reveal the desired photoresist pattern. After the developing process, the substrates were washed with Milli-Q water thoroughly before being blow-dried in a clean supply of nitrogen. Prior to sealing the microchannels using a thermal plastic, holes were drilled into the glass to create an inlet and outlet for the device and the glass tubing was glued in place.

The flow cell used to refold urea-denatured GFP was 6 cm long and 250 μm high, the main channel was 500 μm wide, the channels leading from ports A and B

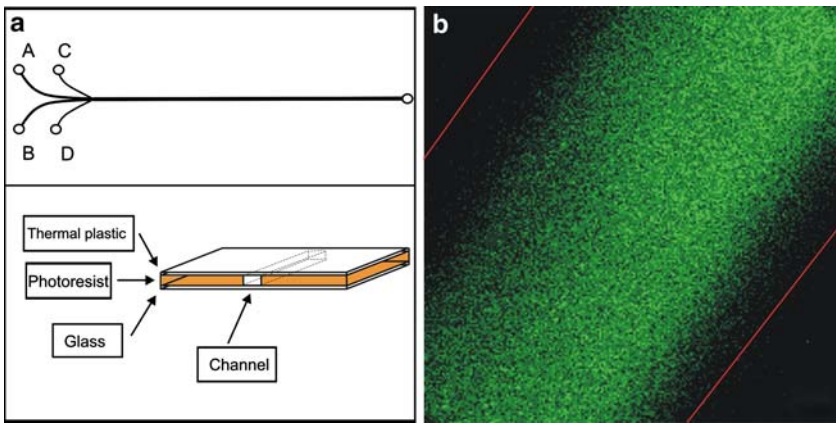

Fig. 1 Confocal images of microfluidic channels where reassembly of urea-denatured GFP is occurring. **a** Schematic diagram of a channel layout as seen from the top and side. **b** Presence of correctly assembled GFP, approximately 1 cm down the microfluidic channel, showing the presence of correctly assembled GFP viewed by confocal microscopy. The denatured GFP was added through port *C* and the refolding solution through port *A*; ports *B* and *D* were blocked off. The channel's edges are indicated in *red*

were 250 μm wide and the ports leading from ports C and D were 100 μm wide (Fig. 1a). Reagents were delivered under laminar flow conditions using a syringe pump delivering 5 μl min^{-1} refolding buffer and 1 μl min^{-1} denatured GFP. Fluorescence was observed using a Leica TCS-NT-UV confocal laser-scanning microscope.

Capillary device assembly

The capillary reactor for refolding BR was constructed using 0.8 mm I.D. PEEK tubing (Sigma Aldrich, Poole, UK) connected using a Y-piece constructed from Perspex, designed to minimise dead space. An Amersham UPC-900 in-line monitor, set to measure absorption at 280, 400 and 550 nm, was used to monitor BR folding. It was fitted 11 cm downstream of the Y-piece, giving a total reactor volume of 55.3 μl. Pumping was by a syringe pump, delivering equal flows of refolding buffer and of denatured protein solution into the reactor.

Expression, purification, denaturation and refolding of GFP

Recombinant GFP was expressed as glutathione S-transferase (GST) fusion protein (vector pGEX-5X-1, Pharmacia) in the BL21 strain of *E. coli*. In the case of acid denaturation, the GST-fusion GFP was purified using glutathione Sepharose (Amersham) with 10 mM of reduced glutathione and denatured by diluting in an equivalent volume of buffer AD [29 mM Tris, 147 mM NaCl, 0.74 M L-arginine, 0.74 mM ethylenediaminetetraacetate (EDTA), 1.47 M HCl]. For urea-denaturation, GFP was directly cleaved from the glutathione Sepharose column with factor Xa in buffer UC (20 mM Tris pH 7.3, 200 mM NaCl, 5 mM $CaCl_2$). An equal volume of this GFP solution was added to buffer UD [8 M urea, 20 mM Tris pH 8.14, 10 mM EDTA, 200 mM NaCl, 10 mM dithiothreitol (DTT)] and heated at 95°C for 5 min (final protein concentration 0.16 mg ml^{-1}). In each case, the subsequent lack of fluorescence confirmed that the protein was denatured.

The acid-denatured GFP was subsequently refolded by mixing with an equal volume of buffer AR (765 mM Tris pH 8.0, 147 mM NaCl, 0.74 M L-arginine, 0.74 mM EDTA). The urea-denatured GFP was refolded by mixing with 5 volumes of buffer UR (20 mM Tris pH 7.4, 200 mM NaCl, 10 mM DTT).

Purification, denaturation, and refolding of BR

Wild-type BR was prepared from purple membrane of *H. salinarium* and denatured in SDS, as previously described by Oesterhelt and Stoeckenius (1974) and Booth et al. (1996). The protein was at first delipidated by adding 1 ml of purified purple membrane to 30 ml of buffer B [chloroform/methanol/triethylamine (100/100/1)] and centrifuged at 8,000 rpm for 20 min. After discarding the supernatant, this process was repeated twice. After a final centrifugation of 8,000 rpm for 1 h, the supernatant was again discarded and the pellet was left to air-dry. The pellet was then resuspended in 2 ml of 1% SDS and the absorption spectrum measured confirmed that the retinal had been fully dissociated from the protein. The final protein concentration was 0.1 mg ml^{-1}. BR was then refolded by mixing with an equal volume of buffer LR [20 mM Tris pH 7.4, 250 mM NaCl, 2% dimyristoyl-phosphatidylcholine (DMPC), 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS), 2.5 mM retinal, sonicated for 1 h]. The extinction coefficient for unfolded BR in SDS was 66,000 cm^{-1} M^{-1} at 280 nm. The concentration of refolded BR was determined using an extinction coefficient of 52,000 cm^{-1} M^{-1} at 551 nm (Greenhalgh et al. 1993).

Background

Microfluidics is the term used to describe flow in devices capable of handling volumes of fluid in the range of nano- to microlitres. Mixing (or lack thereof) is often crucial to the effective functioning of these devices. Much research has focused on enabling rapid mixing through turbulence (Nguyen and Wu 2005). However, it is also possible to produce stable laminar flows: at sufficiently low flow rates, two initially segregated streams would co-flow side by side, and the mixing would occur by molecular diffusion alone. The time for a particle to diffuse across the full width of a microfluidics channel, which we define as *mixing time scale* of the particle, would therefore provide a good estimate of the time required for complete mixing to occur.

It was shown by Albert Einstein that for the diffusion of a particle, the mean square displacement in a particular direction, $\langle x^2 \rangle$ is proportional to time t (Einstein 1905),

$$\langle x^2 \rangle = 2D \times t \quad (1)$$

where D, the particle's diffusion coefficient, is of the order of 10^{-7} cm^2 s^{-1} for proteins like GFP [0.5–8.7×10^{-7} cm^2 s^{-1} (Dayel et al. 1999)], 6.0×10^{-7} cm^2 s^{-1} for DMPC/CHAPS micelles (Andersson and Maler 2005) and of the order of 10^{-5} cm^2 s^{-1} for small molecules like urea [1.4×10^{-5} cm^2 s^{-1} (Gosting and

Akeley 1952)] and SDS [0.06–0.5 × 10^{-5} cm^2 s^{-1} (Orfi et al. 1998)].

Protein refolding is often initiated by the removal of the much smaller denaturant, the much slower diffusion rate of the protein can be disregarded and we need to focus only on the diffusion of the smaller molecules. The *mixing time scale*, t_D, would therefore be equal to $a^2/2D$, where a is the width of the channel. Assuming a diffusion coefficient of the order of 10^{-5} cm^2 s^{-1} for small molecules (both denaturing and renaturing) and a typical channel width of 0.5 mm, we obtain *mixing time scales* $t_D \sim 125$ s.

Mixing during integral membrane protein refolding in lipid–detergent micelles is more complex, as the individual diffusion coefficients for both lipid and detergents (CHAPS and SDS) would be directly affected by their individual concentrations, as well as by the composition of the renaturation buffer. A simple mechanism of bidirectional diffusion of protein and of DMPC/CHAPS micelles across the channel width would nevertheless result in mixing under 10 min.

Residence times of 10 min or more within the microfluidics device would therefore provide sufficient time for the protein to transfer from a denaturing solution to the refolding buffer. This duration is much slower than refolding protein by dilution, but much faster than times typically involved in refolding by dialysis. In the latter case, small solutes would require approximately 14 h to fully diffuse out of a bag of 1 cm radius.

Results and discussion

Refolding of urea-denatured GFP

Green fluorescent protein was denatured using 8 M urea at 95°C for 5 min, which eliminated its fluorescence. Larger-scale refolding in a cuvette indicated that most fluorescence was recovered after 10–15 min, suggesting that similar residence times in the microfluidic channel would be required. Refolding was subsequently achieved within a glass-enclosed microfluidic channel, where the correctly assembled GFP could be observed with the aid of a confocal microscope (Fig. 1). The device's main channel contained the refolding buffer and a smaller side channel contained denatured GFP (Fig. 1a). The fluid streams flowed in a laminar manner down the channel and refolding of the GFP occurred as the urea diffused away. This was observed as a uniform stream of fluorescence further down the microchannel. The uniformity of the GFP stream indicated that the protein was not aggregating or binding to the channel walls (Fig. 1b).

Reassembly of acid-denatured GFP by neutralisation of the pH was observed to form a very uneven fluorescence indicating that a degree of aggregation was taking place (supplementary figure). The aggregates were not observed to interfere with the fluid flow along the channel, indicating that blockage of the device due to aggregation is unlikely to be a problem.

Folding of BR

This microfluidics approach was consequently extended to refolding integral membrane proteins. In the refolding reactor, the denatured protein is transferred from a detergent solubilising environment into a lipid-rich environment, which enables the protein to correctly refold due to its interaction with surrounding lipids and detergents (Booth et al. 1996; Sugiyama and Mukohata 1996).

In order to study the rate of BR folding, the SDS-denatured BR and the folding mixture, containing DMPC, CHAPS and retinal, were flushed through the capillary reactor at different flow rates (Fig. 2a). BR was shown to fold under these conditions as shown in Fig. 2b.

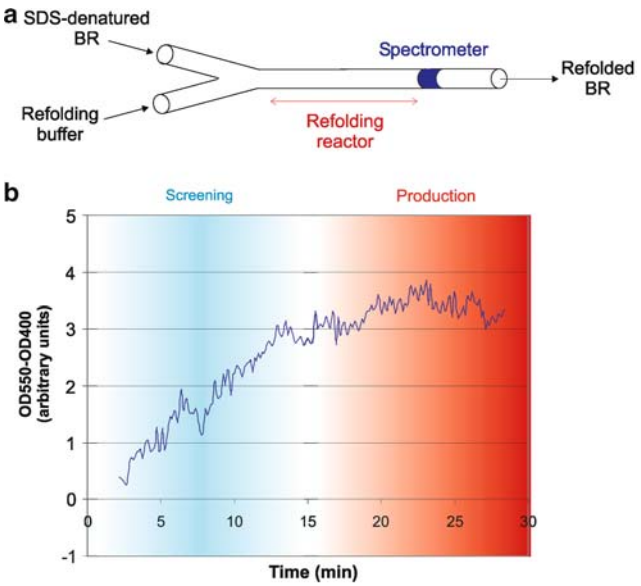

Fig. 2 **a** Schematic diagram of the capillary reactor used for refolding BR. **b** BR refolding within a capillary reactor (11 cm long with a 0.8 mm diameter) detected as a shift in absorption at 550 nm, with respect to absorption at 400 nm, by an inline spectrometer. The screening phase, which can assess if refolding is occurring, is followed by the production phase, when the refolding reactor's operation has reached a stable state

The flow rate was varied in order to measure refolding yields associated with different residence times in the microfluidics reactor (Fig. 3a). The data suggests that in the steady-state *production* mode, the kinetics associated with the first 60 min of folding closely followed that seen in larger-scale experiments in a mixed cuvette (the former had rate constants of ~0.04 min^{-1}, compared to ~0.09 min^{-1}). The resultant refolded protein had similar optical absorption characteristics as that of the native BR from the purple membrane of *H. salinarium* [OD_{280}/OD_{550} ~ 3.4–3.8 for refolded BR (>30% yield) against OD_{280}/OD_{550} ~ 1.9 for native BR (70% yield)].

If the folding rate were dominated by the rate of diffusion between the fluid streams or by the kinetics of folding alone, the flow rate through the reactor would not be expected to affect the folding rate. The role of flow rate on BR folding was assessed and was found to have an important effect on the initial rate of folding (Fig. 3b). The reactor was rapidly filled and subsequently emptied at different flow rates. The initial rate of folding was strongly influenced by the flow rate through the reactor, with the higher folding rate being linked to slower flow rates. The rational behind the observed influence of flow rate on folding rate is not obvious. The observation may be due to the effect the flow rate has on the lipid–detergent micelles, rather than directly on BR folding, and due to the interaction between the micelle and the wall of the tubing. However, from a *screening* standpoint, it is clearly possible to rapidly identify successful refolding conditions at flow rates under 3 μl min^{-1}.

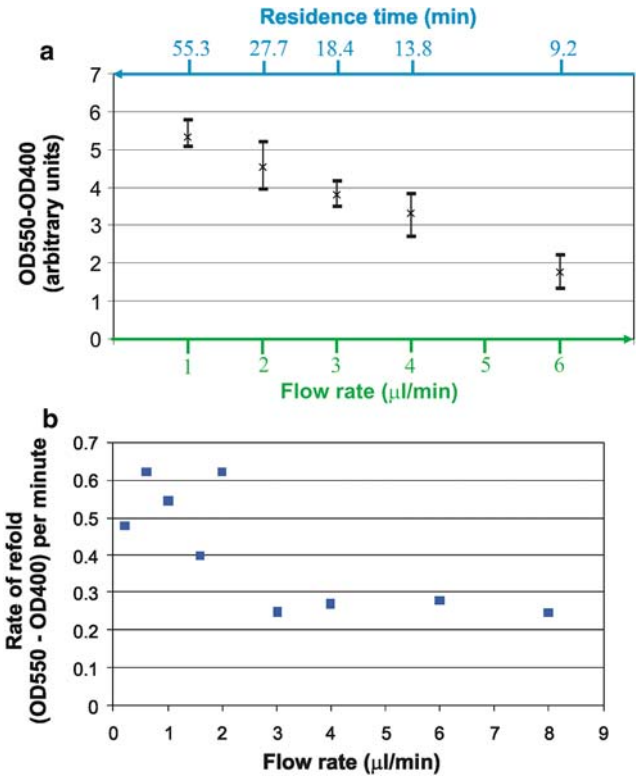

Fig. 3 **a** Refolding yields of BR according to residence time within the capillary reactor. The yields' mean and observed variations during the production phase are shown. **b** Refolding rates of BR according to flow rates within the capillary reactor during the screening phase

Conclusion

The work described in this paper illustrates that microfluidic channels can be used as reactors for refolding soluble and transmembrane proteins. Observation of fluorescence using confocal microscopy enabled GFP refolding to be demonstrated as well as observing phenomenon within the reactor, such as any interaction with channel walls and aggregation taking place. The work with BR illustrated that this technology could also be applied to refolding transmembrane proteins. The discovery of the role played by the flow rate on the BR folding rate, however, indicates that there is still much to understand about this technique, especially when folding the more complex transmembrane proteins.

These data suggest that this method can potentially open to study a diverse range of proteins. A modular system can in principle be easily implemented. The addition of other fluid streams would enable incremental, time-controlled mixing of different renaturing agents with the protein. The transmembrane region of a protein could, for example, be refolded prior to its soluble domain. Moreover, this type of refolding is principally controlled by the diffusion of the denaturing and renaturing molecules and hence not limited by the size of the protein. Further study would however be required to assess its suitability for refolding polymeric proteins, like myelin and tubulin; in particular, whether the physical constraints of a microfluidics system would be able to define polymer length, as previously observed during the synthesis of the polymer poly (2-hydroxypropyl methacrylate) (Wu et al. 2004).

The microfluidic refolding reactor has the flexibility to be used in two modes of operation. To screen folding conditions, the system can sequential process the protein under different refolding conditions. The effects of different lipids, detergents, salts and pHs can therefore be rapidly assessed. Once an optimum refolding solution has been identified, the reactor can subsequently be operated continuously to generate

sufficient amounts of protein for crystallisation trials. It would currently be able to process >0.15 mg of GFP and >0.10 mg of BR overnight. This work presents the first step in developing a functional microfluidics protein refolding device for high-throughput applications in structural genomics research.

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