Shear-Stress-Mediated Refolding of Proteins from Aggregates and Inclusion Bodies

Colin L. Raston,*[e] and Gregory A. Weiss*[a, c]

Recombinant protein overexpression of large proteins in bacteria often results in insoluble and misfolded proteins directed to inclusion bodies. We report the application of shear stress in micrometer-wide, thin fluid films to refold boiled hen egg white lysozyme, recombinant hen egg white lysozyme, and recombinant caveolin-1. Furthermore, the approach allowed refolding of a much larger protein, cAMP-dependent protein kinase A (PKA). The reported methods require only minutes, which is more than 100 times faster than conventional overnight dialysis. This rapid refolding technique could significantly shorten times, lower costs, and reduce waste streams associated with protein expression for a wide range of industrial and research applications.

Overexpressed recombinant proteins for industrial, pharmaceutical, environmental, and agricultural applications annually represent a more than $160 billion world market.[1] Protein expression for a wide range of industrial and research production of proteins. In this method, a glass cylinder (10 mm by 16 cm) is spun rapidly (5 krpm) at a 45° angle. At high rotational speeds, the solution within the sample tube forms micrometer-thick, thin fluid films, which flow with the same speed and direction as the wall of the glass tube. The rotating glass tube generates a velocity gradient within the thin fluid film, which introduces shear stress into the solution (Figure 1 A). We imagined applying the VFD with a similar range of input energies to the refolding of proteins: the device produces unusual shear within such films and has been shown to be effective in disassembling molecular capsules[11] and reducing the size of micelles used in the fabrication of SBA-15 mesoporous silica with control over the pore size of the material.[12] Importantly, the latter occurs at room conditions.[10–12] Recovery of correctly folded proteins from aggregates is inefficient and challenging for large-scale industrial processes. Mechanical methods to solve this problem have been reported. One approach applies very high hydrostatic pressures (400 bar) to refold recombinant proteins from inclusion bodies.[13–16] We report a novel method by applying finely controlled levels of shear stress to refold proteins trapped in inclusion bodies. This method might be capable of broadening the utility of bacterial overexpression, and could transform industrial and research production of proteins.

We report using a vortex fluid device (VFD) to apply shear forces for rapid equilibration of protein folding and isolation of intermediates during protein folding. In this method, a glass cylinder (10 mm by 16 cm) is spun rapidly (5 krpm) at a 45° angle. At high rotational speeds, the solution within the sample tube forms micrometer-thick, thin fluid films, which flow with the same speed and direction as the wall of the glass tube. The rotating glass tube generates a velocity gradient within the thin fluid film, which introduces shear stress into the solution (Figure 1 A). We imagined applying the VFD with a similar range of input energies to the refolding of proteins: the device produces unusual shear within such films and has been shown to be effective in disassembling molecular capsules[11] and reducing the size of micelles used in the fabrication of SBA-15 mesoporous silica with control over the pore size of the material.[12] Importantly, the latter occurs at room

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201402427.
temperature rather than requiring hydrothermal processing and suggests that the constant "soft" energy in the thin film has application in manipulating macromolecules, such as the refolding of proteins, without requiring heating. Other relevant applications and optimization of a similar VFD include exfoliating graphite and hexagonal boron nitride to generate mono- and multilayer structures,[17, 18] controlling the formation of different calcium carbonate polymorphs,[19] and when controlling chemical reactivity and selectivity.[20, 21]

Modeling the fluid behavior in the VFD allowed estimation of the shear forces experienced by proteins folding at various rotational speeds. Our analysis applies the solution for cylindrical Couette flow.[20, 21] The velocity of the solution, \( v_0 \), is a function of the radius, \( r \) (Figure 1A). The boundary conditions for the liquid film interfaces are defined as follows. The inner air–liquid interface at \( r = R_1 \), slips, due to discontinuity in viscosity, and results in increasingly low shear stress (\( dv_0/dr = 0 \)). At the outer liquid–glass interface, the no-slip boundary dictates that the velocity of the liquid at \( r = R_2 \) matches that of the inner wall of the glass tube (\( v_0 = R_2 \Omega \), where \( \Omega \) is the angular velocity of the tube. The resulting velocity profile is a nonlinear function of the form

\[
v_0 = Ar + B/r \]

where

\[
A = \frac{\Omega}{R_1^2 + 1} \quad \text{and} \quad B = \frac{\Omega R_1^2}{R_2^2 + 1}.
\]

From this velocity profile, shear stress can be calculated as

\[
\tau_\theta = \mu \frac{\partial}{\partial r} \left( \frac{v_0}{r} \right)
\]

where \( \mu \) is the viscosity of water at 20 °C. At a speed of 5 krpm, the calculated shear stress ranges from 0.5246 to 0.5574 Pa (Figure 1B). Although this model provides insight into the shear stress range, the fluid flow will be perturbed by the cross vector of gravity with centrifugal force for a tube oriented at 45°.[20] which might also facilitate protein folding; this angular setting also proved optimal for the aforementioned applications of the VFD. The calculated values of shear stress are similar to the levels previously reported to cause protein unfolding,[20] and we hypothesized that short periods of VFD processing could allow equilibration of protein unfolding.

Experiments with native hen egg white were conducted to determine if shear forces could refold denatured hen egg white lysozyme (HEWL) in complex environments. The separated white lysozyme activity, was 44 ± 4 U/mL, for 20 min. The resultant hard-boiled egg white was dissolved in 8M urea, rapidly diluted into PBS (1:100), and then VFD-processed at the indicated rotational speeds and times (Table S1 and Figure 2A). The total protein concentration, as determined by bicinchoninic acid assay, was 44 μg/mL. The recovery of HEWL activity was then determined by a lysozyme recovery assay (Figure S1). Refolding HEWL within the egg white at 5 krpm recovered glycosidase activity even after a short 2.5 min spin, but continued shear forces unfolded the protein. As mentioned above, shear-stress-mediated protein unfolding has been observed for other proteins previously. Optimization of VFD times, speeds, and concentration of chaotropic additives is required to find conditions allowing re-equilibration of protein unfolding without causing loss of the protein structure. VFD processing for 5 min at 5 krpm in 1M urea resulted in optimal HEWL refolding as monitored by lysozyme activity (Figure 2A). This experiment establishes the basic parameters for protein refolding by VFD.

To demonstrate refolding of recombinantly expressed, reduced HEWL, the cell pellet was first reconstituted in lysis buffer containing 2-mercaptoethanol, denatured in 8 M urea, and rapidly diluted into PBS (1:100). Buffers used for the expression and purifications of all recombinant proteins are described in Table S2. Second, the diluted protein (1 mL, 44 μg/mL) protein) was immediately transferred to the VFD sample tube and spun at 22 °C and 5 krpm for 5 min. Circular dichroism (CD) spectra of the VFD-refolded, recombinant HEWL demonstrated restoration of secondary structure from proteins isolated from inclusion bodies. After VFD processing, the CD spectra of identical HEWL samples demonstrated partial recovery of secondary structure compared to the native lysozyme (Figure 2B). Yields of functional protein determined by lysozyme activity assay are shown in Figure 2D.
HEWL could also be refolded by continuous-flow VFD. This approach delivers additional sample through an inlet at the cylinder base. The sample (50 mL), added at a flow-rate of 0.1 mL min⁻¹, demonstrated significant recovery of HEWL activity for scalable, high volume applications (Figure 2C). The recombinant HEWL recovered > 82% of its activity following VFD treatment. As expected, HEWL isolated from inclusion bodies without VFD processing failed to show any lysozyme activity (Figure 2D). The continuous-flow approach could be readily scaled up and parallelized for industrial applications requiring treatment of very large volumes.

After refolding denatured lysozyme in both complex (egg white) and simple (purified recombinant protein) environments, the next experiments focused on refolding the protein caveolin-1, as an example of a protein requiring an inordinate amount of processing time by conventional approaches (e.g., four days of dialysis). A caveolin variant without its transmembrane domain (caveolin-ΔTM) was recombinantly expressed, and the inclusion body was purified under denaturing conditions. Purified caveolin-ΔTM was diluted and then underwent a short (1 h) dialysis to decrease the urea concentration to 1 M. The protein was then VFD-treated for 0, 10, or 30 min at 5 krpm at a concentration of 186 mM. The CD spectra of the VFD-treated caveolin-ΔTM showed pronounced minima at 208 and 220 nm, which are indicative of α-helical secondary structure.[23] Solution turbidity also decreased sharply following VFD treatment, which illustrates VFD solubilization and refolding of partially aggregated proteins (Figure 3B). ELISA experiments examined binding by the refolded caveolin-ΔTM to HIV glycoprotein 41 (gp41), a known caveolin binding partner.[24–26] VFD processing significantly restored protein function, as shown through binding to gp41 (Figure 3C).

Larger-sized proteins initially failed to refold, despite VFD treatment. For example, the catalytic domain of CAMP-dependent protein kinase A (PKA, 42 kDa) is significantly bulkier than HEWL (14 kDa) and caveolin-ΔTM (17 kDa) and did not refold from inclusion bodies after treatment with similar protocols to the above experiments. To refold full-length PKA in vitro, we adapted the VFD to provide a closer mimic of cellular folding. In cells, the nascent polypeptide can fold as the N terminus extrudes from the ribosome, whereas in vitro refolding must address the entire protein at once.[27] Thus, we attempted to focus shear stress on the N terminus of His-tagged PKA by binding the unfolded protein to Ni²⁺-charged immobilized metal affinity chromatography (IMAC) resin. The His₆-PKA-IMAC complex (1 mL, 0.2–1 mg mL⁻¹) was then subjected to refolding by VFD. Following VFD treatment, His-PKA separated from the IMAC resin and recovered 69% of its kinase activity (Figure 4). Interestingly, the remaining His-PKA eluted from the IMAC resin by imidazole yielded far less active protein (Figure S3). We hypothesize that protein folding makes the His₆ epitope less accessible for strong binding to the IMAC resin subjected to shear by the VFD. Under conventional conditions, IMAC resin binds to folded PKA, but we recovered protein remaining on the IMAC resin after VFD treatment to demonstrate its lack of folding and catalytic activity (Figure S3). Thus, as the VFD folds the protein, PKA elutes from the affinity resin. Negative control experiments with identical conditions but with uncharged IMAC resin, decreased quantities of charged resin, or 500 mM imidazole to block the Ni²⁺-His₆ tag interaction showed only low levels of kinase activity (Figure 4).

As reported here, protein refolding by VFD requires optimization for each protein. Buffers, chaotrope additive, protein concentration, and processing time were optimized for HEWL, caveolin-ΔTM, and PKA. The refolding of HEWL from the complex mixture of boiled egg whites appeared less efficient than recovery of the folded protein isolated from inclusion bodies. In egg whites, the mechanical energy of the VFD could be misdirected to the other >95% of proteins present.

This report offers significant advantages over conventional approaches to protein refolding. First, VFD-mediated refolding requires much smaller solution volumes (ca. 1% of the volumes required for conventional dialysis). Second, this key step in protein production occurs more than 100 times faster than overnight dialysis, with >1000-fold improvements for proteins such as a caveolin. Notably, introducing high shear in thin fluid
films is a low energy, inexpensive process and can be readily implemented for relatively low cost in conventional biochemistry laboratories.

The advantages of VFD refolding open new possibilities for increasing protein yields in simple cell lines. By using the constant soft energy of the thin microfluidic films, the VFD can untangle complex mixtures, aggregates, and insoluble inclusion bodies. In industrial and academic applications, high concentrations of a chemical inducer like IPTG could drive overexpressed proteins into insoluble inclusion bodies before VFD-based refolding. Conversely, most conventional processes avoid inclusion bodies by optimizing growth conditions and special cell lines at the expense of higher yields and purer protein isolated directly from bacterial cells. As reported here, the continuous-flow mode of the VFD or parallel processing with multiple VFD units could allow scale-up to accommodate much larger solution volumes. Thus, the approach could drastically lower the time and financial costs required to refold inactive proteins on an industrial scale. The VFD sample tube itself can also be modified to amplify or otherwise direct the intensity of shear forces applied. For example, modified surfaces with high contact angle and/or with textured features could enhance the turbulent flow, altering the applied shear stress. Harnessing shear forces to achieve rapid equilibration of protein folding could be expanded to a wide range of applications for research and manufacturing.

Acknowledgements

We thank Professor Abraham Lee for discussion and advice on fluid modeling. This work was supported by the National Institute of General Medical Sciences of the NIH (R01 GM100700-01) and the Australian Research Council (ARC DP1092810 and DP13010066).

Keywords: green chemistry · protein expression · protein folding · shear stress


Received: July 30, 2014
Revised: December 1, 2014
Published online on January 23, 2015

Figure 4. Facilitating VFD refolding of PKA by pre-binding the His6-tagged protein to IMAC resin. PKA (1.7 mg) was pre-incubated with 250 or 50 μL IMAC resin in 6 mM guanidine-HCl prior to dilution to 1 mM guanidine-HCl and VFD treatment. Excess PKA was removed by an imidazole (1 mM) wash buffer. The PKA activity per μg of protein was measured by a nicotinamide adenine dinucleotide (NADH) enzyme-linked assay and is shown here as a percentage of untreated, wild-type PKA activity (WT). Dark gray bars denote samples that are VFD-treated. In this assay, the consumption of ATP by PKA catalysis results in a lactate dehydrogenase-coupled decrease in the levels of NADH, monitored through measuring absorbance at 340 nm. The control experiments included low quantities of resin (50 μL IMAC) and two controls for non-specific binding to the resin in the absence of the Ni2+ His6 tag interaction (500 mM imidazole added, and uncharged IMAC resin lacking Ni2+). Imidazole was diluted to 50 mM before the kinase assay.

Acknowledgements

We thank Professor Abraham Lee for discussion and advice on fluid modeling. This work was supported by the National Institute of General Medical Sciences of the NIH (R01 GM100700-01) and...