Urea and GdnHCl are typical protein destabilizers (denaturants). At high concentrations, they mostly denature proteins by the chaotropic effect. In contrast, it is known that, at low denaturant concentrations, they tend to stabilize the structure of the target protein, leading to inhibition of protein aggregation during refolding [21,22]. Although the mechanism remains unclear, at low concentrations the aggregation rate markedly decreases compared with the refolding rate. $(NH_4)_2SO_4$ is a protein stabilizer that can stabilize the protein structure at low concentration through electrostatic interactions, which changes the solubility of the native (folded) structure protein. However, protein destabilizers often accelerate protein aggregation. Some amino acids are classified as protein aggregation inhibitors, including arginine and its derivatives. Recently, this type of chemical additive has been widely used for the refolding process to increase refolding yields by decreasing aggregation.

Due to these interesting features of protein refolding, chemical additives are often added to the refolding buffer in the dialysis and dilution methods to decrease the degree of aggregated and/or misfolded proteins. Thus, many additives have been developed and used to improve the recovery yields of the active proteins [23]. In many cases, the inclusion body proteins have been successfully refolded. In this section, we introduce inhibitors and stabilizers (Figure 2) and discuss the application of the additives, and, then, discuss artificial chaperone-assisted refolding.

3.1. Amino Acids

The addition of arginine hydrochloride (ArgHCl) or arginine (Arg) to the refolding buffer has been reported to have interesting effects during the refolding process, such as improving the solubility of proteins and inhibiting protein aggregation. Thus, Arg has been studied as an additive for refolding of denatured model proteins [14,24,25] and has been applied for the refolding of a variety of proteins from inclusion bodies, such as casein kinase II [26], gamma interferon [27], p53 tumor suppressor protein [28], and interleukin-21 [29].

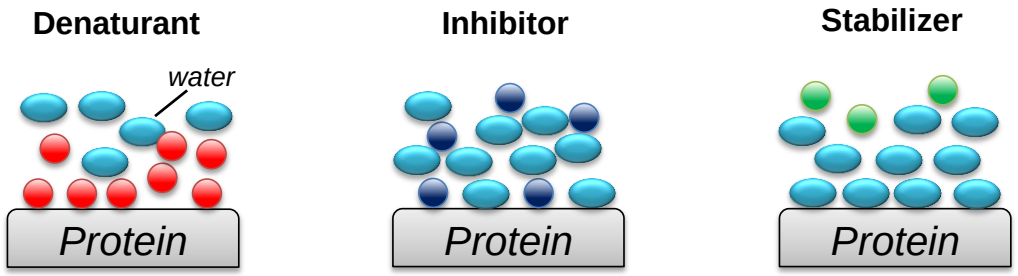
Despite its effectiveness, the molecular mechanism of Arg is still unclear. ArgHCl has a guanidinium group (Gdn) at the distal end of its side chain, which is similar to GdnHCl (Figure 2). Gdn strongly interacts with hydrophobic amino acids in hen egg-white lysozyme [30] and acts as a strong chaotropic reagent. The crystal structure of the lysozyme–Arg complex showed that Arg bound at sites different from those of Gdn, and mildly interact with nearby hydrophobic amino acid of the protein surface [31]. These interactions included electrostatic, hydrophobic, and cation–π interactions. Compared with Gdn, the amino and carboxyl groups of Arg form weaker hydrogen bonds with the denatured protein and water (Figure 3). Thus, Arg might act as an aggregation inhibitor due to its moderate binding to proteins [32].

In addition to Arg, other amino acids have been reported to be moderate chaotropic agents. Arginineamide and glycineamide have been reported to be aggregation inhibitors for protein refolding and increase the refolding yields of the proteins better than Arg [32–34]. Although their moderate chaotropic effects are unclear, the crystal structure of the lysozyme–glycine amide complex indicated that glycine amide binds to the protein surface near hydrophobic residues, such as aromatic residues, decreased the amount of bound water, and increased the mobility of the protein [34]. In addition, the binding sites of glycineamide were different from those of Arg, and almost all of the bound glycine amide only formed hydrogen bounds between the carboxyl amide group and surface residues of the

protein and hydrated water [34]. These results support that the different binding sites of glycine amide and Arg probably lead to their different inhibitory abilities.

Proline has also been reported to enable proteins refolding to their native (active) conformation. It was proposed that proline inhibits protein aggregation by binding to the folding intermediate(s) and trapping the folding intermediate(s) in the supramolecular assembly with proline [35].

Figure 3. Models of the interactions between the surface of the protein with water and chemical additives. The additives and molecular water are represented by colored circles and ellipses, respectively: denaturant (strong chaotropic reagent), red; inhibitor (moderate chaotropic reagent), dark blue; stabilizer (osmotic reagent), green; and water, light blue.



3.2. Glycerol, Polyethylene Glycol and Sugars

Glycerol acts as a protein stabilizer (Figures 2 and 3) by enhancing the hydrophobic interactions as a consequence of an increase in the solvent ordering around the proteins. Increasing the glycerol concentration increases the stability of proteins even at high protein concentrations [36,37]. Similarly, polyethylene glycol [38,39] and sugars [40,41] have been reported to act as stabilizers of protein structures. For example, polyethylene glycol has been successfully used for refolding of insulin-like growth factor [42] and interferon [43]. During the refolding process, these additives bind to the intermediate in the folding pathway of the protein or interact with the hydrophobic side chains of the denatured protein. It has been suggested that these additives create an ideal environment where the refolding rate increases while the aggregation rate decreases [36–41]. Although such stabilizers increase the refolding yields, protein aggregation simultaneously occurs. Therefore, these types of additives have always been used in combination with an aggregation inhibitor such as Arg [37,42].

3.3. Cyclodextrins

Cyclodextrins have been reported to inhibit protein aggregation during the refolding process [44–46]. Cyclodextrins have an amphipathic structure. The hydrophobic cavities and hydrophilic groups (mainly hydroxyl group) can interact with proteins by weak interactions, such as hydrogen bonds, van der Waals interactions, and hydrophobic interactions, and probably have a similar moderate chaotropic effect to ArgHCl.

In addition to their effect as a protein aggregation inhibitor, cyclodextrins have been used for other refolding methods. In host–guest chemistry, it is well known that cyclodextrins, such as β-cyclodextrin, can capture chemical compounds in their central hydrophobic cavities. Based on this property, cyclodextrins have been used as stripping agents for the removal of detergents from denatured protein

by surfactants. This refolding method is called artificial chaperone-assisted refolding [47,48]. The method is based on chaperonin proteins in bacteria. Bacterial GroEL and GroES proteins (chaperonin) are molecular chaperones for protein refolding *in vivo*. In this system, misfolded protein is captured in the hydrophobic central cavity by the GroEL tetradecamer. When the GroES heptamer caps the cavity, protein refolding occurs in an ATP-dependent manner. The artificial chaperone-assisted refolding method mimics the GroEL-GroES system [47,48]. The refolding involves two steps. In the first step, the denatured protein is diluted with a buffer containing detergents that prevent protein aggregation. In the second step, the protein–detergent complex solution is diluted with a buffer containing cyclodextrins that strip detergent from the complex. Various cyclodextrin derivatives and detergents have been synthesized for effective protein refolding [49]. A large variety of proteins have been refolded by the artificial chaperone-assisted refolding methods [47–49].

4. Decreasing Denaturant Concentration by Laminar Flow in Microfluidic Chips

It has been suggested that the refolding procedure in a short period of time may reduce the formation of protein aggregates and achieve efficient protein refolding [19,20]. However, it is difficult to efficiently refold proteins in a short time using the conventional methods discussed in Sections 2 and 3. Although the chemical additives described in Section 3 are helpful to inhibit protein aggregation, the procedure usually requires multiple days. To overcome this difficulty, we have proposed the microfluidics approach as a rapid and simple refolding method [15].

Microfluidic reaction systems are widely studied and used in chemistry and biotechnology fields [50–53]. The laminar flow in microchannels can be used to create a well-defined and predictable interfacial region among the streams. Additionally, diffusion mass transfer is enhanced in the microchannel compared with in the macrochannel. These characteristics inspired us to control the gradual removal of denaturants from chemically denatured proteins in the laminar flow. Previous reported microfluidic chips for refolding were designed to study the initial folding events during rapid mixing of the denatured protein solution and refolding buffer through either turbulent flow [54,55] or diffusion [56,57]. In these kinetic studies, easy-to-fold proteins where the measured protein folding occurs within 100 μs [54], such as cytochrome c, were used. In contrast, difficult-to-fold proteins, which often form inclusion bodies, aggregate in the microchannel by rapid mixing [58] due to rapid removal of denaturant from the denatured proteins. It suggests that microfluidic chips with rapid mixing are not applicable for refolding of difficult-to-fold proteins.

4.1. Design of Microfluidic Chips

Gradual decrease of denaturant concentration from the denatured protein within a short period of time may lead to efficient protein refolding. The concept of protein refolding using microfluidic chips is based on controllable diffusion of the denaturant from the denatured protein stream to the diluting buffer in laminar flow. A similar concept of controllable diffusion of reagents by hydrodynamic flow has been applied for the synthesis of polymeric nanoparticles [59] and for rapid protein concentration analysis [60].

In laminar flow in the designed chips (Figure 4a), the fluid stream to be mixed flows along the central stream (denatured protein) and encounters two buffer streams at junction *a*. In general, the mean square displacement (<x^2>) of molecules in solution is proportional to mixing time *t*:

$$< x^2 > = 2Dt \quad (1)$$

where *D* is the diffusion coefficient and is of the order of 10^{-7} cm^2/s for proteins ($0.5 - 8.7 \times 10^{-7}$ cm^2/s) [61] and of the order of 10^{-5} cm^2/s for small molecules like urea (1.4×10^{-5} cm^2/s) [62], indicating that denaturants diffuse two orders of magnitude faster than proteins. In addition, at low Reynolds number (*Re* < 20 under our experimental conditions), the central stream of denatured protein is squeezed into a narrow stream between the two adjacent buffer streams. The width of the focused stream depends on the flow rate of the diluting buffer [59,63]:

$$t = \frac{< x^2 >}{[9D\left(1 + \frac{1}{R}\right)^2]} \quad (2)$$

where *R* is the ratio of flow rate of the denatured protein to the flow rate of the refolding buffer [59]. The denaturant in the central stream of the denatured protein then enables mixing with the buffer by diffusion and the denaturant concentration decreases, meaning that the ratio of flow rates of the refolding buffer can control the denaturant concentration in the microchannel.

Figure 4. Microfluidic chip used for protein refolding [15]. (**a**) Designed microfluidic chips. In MR1, the denaturant concentration around the protein rapidly decreases because of diffusion, which is expected to have a similar mechanism to one-step dialysis and dilution. In MR2, the denaturant concentration shows a step-wise decrease, which is a similar mechanism to step-wise dialysis. The denatured protein was injected into channel *a*. The diluting buffer was injected into channels *b* and *c*; (**b**) Confocal fluorescence microscope image at the junction in MR1 showing laminar flow of the urea stream through the diluting buffer streams. The focused urea stream contains *N*-(4-nitrobenzo-2-oxa-1,3-diazolyl)amine (NBD) as an indicator; (**c**) Relative fluorescence intensities of NBD in the urea stream as a function of the distance from the inlet (position *a*). Flow rates (μL/min) are shown in the graph.

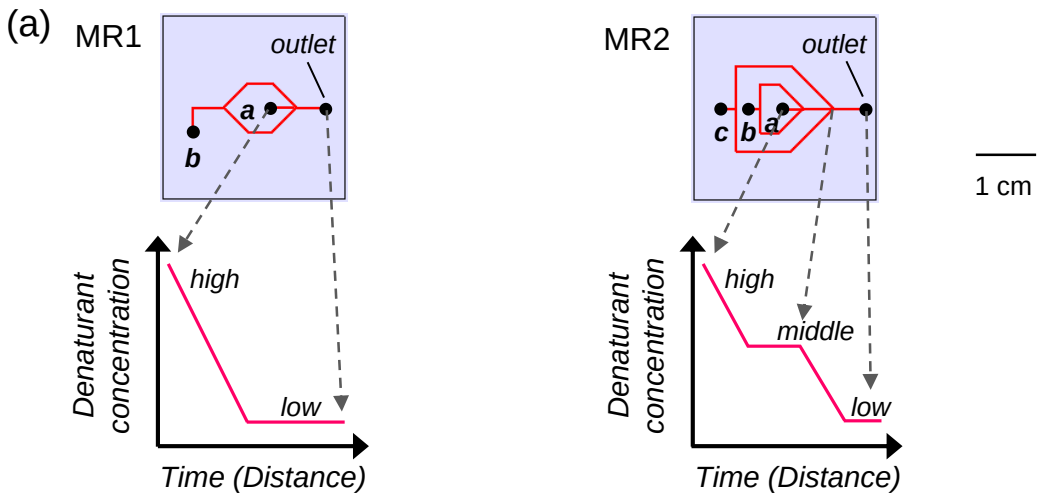
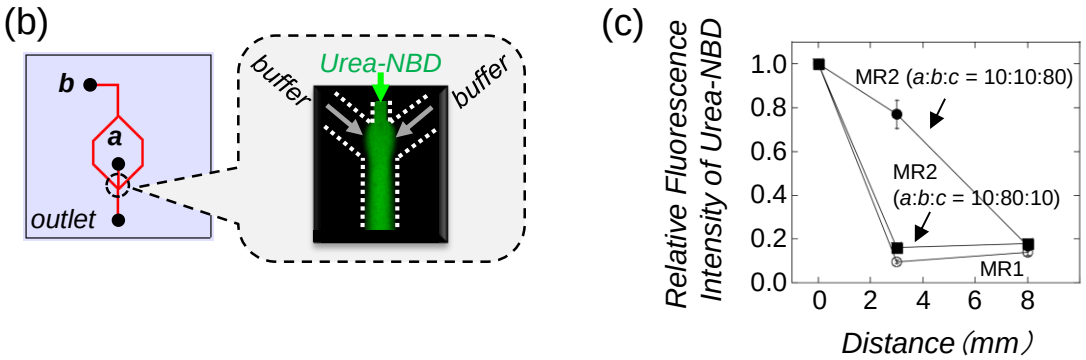


Figure 4. *Cont.*



To refold the protein, the denaturants in the denatured proteins should be diluted at least 10-fold [15]. However, it is expected that a direct 10-fold dilution at the junction in the microchannel may induce misfolding or aggregation [15]. Although varying the flow rate of the diluting buffer can control the distribution of denaturant concentration (Equation 2), a chip with only a single junction cannot generate the gradual decrease of denaturant required for efficient protein refolding. It is expected that better control of the concentration of the denaturant will be achieved by increasing the number of junctions (buffer streams) compared with the chip with only one junction. Therefore, a chip with multiple junctions was designed to generate the gradual decrease of denaturant concentration. Based on these ideas, we designed two types of microfluidic chips: MR1 and MR2 (Figure 4a). The denatured proteins are directly diluted by the buffer in MR1 (one junction), which is expected to have a similar mechanism to one-step dialysis or dilution. On the other hand, MR2 has two junctions that can control the different flow rate ratios of the buffer streams (channels *b* and *c*).

To confirm whether the laminar flow in the designed chips can control the distribution of denaturant concentration, a urea stream in the microchannel was studied by confocal fluorescence microscopy (Figure 4b). As common denaturants, such as urea, do not have a fluorescent property, hydrophilic NBD, which has a similar molecular size and diffusion coefficient to urea, was added to the urea stream as a fluorophore. It is possible that the fluorescence intensity of NBD may be affected by urea concentration. The focus of the microscope was adjusted at position *a* (inlet) in Figure 4a. NBD diluted by the buffer at the junction shows a decrease in fluorescence intensity compared with that at the inlet, indicating that the urea concentration decreased (Figure 4c). The results indicate that the laminar flow in the designed chips can control the distribution of denaturant concentration, as expected [15].

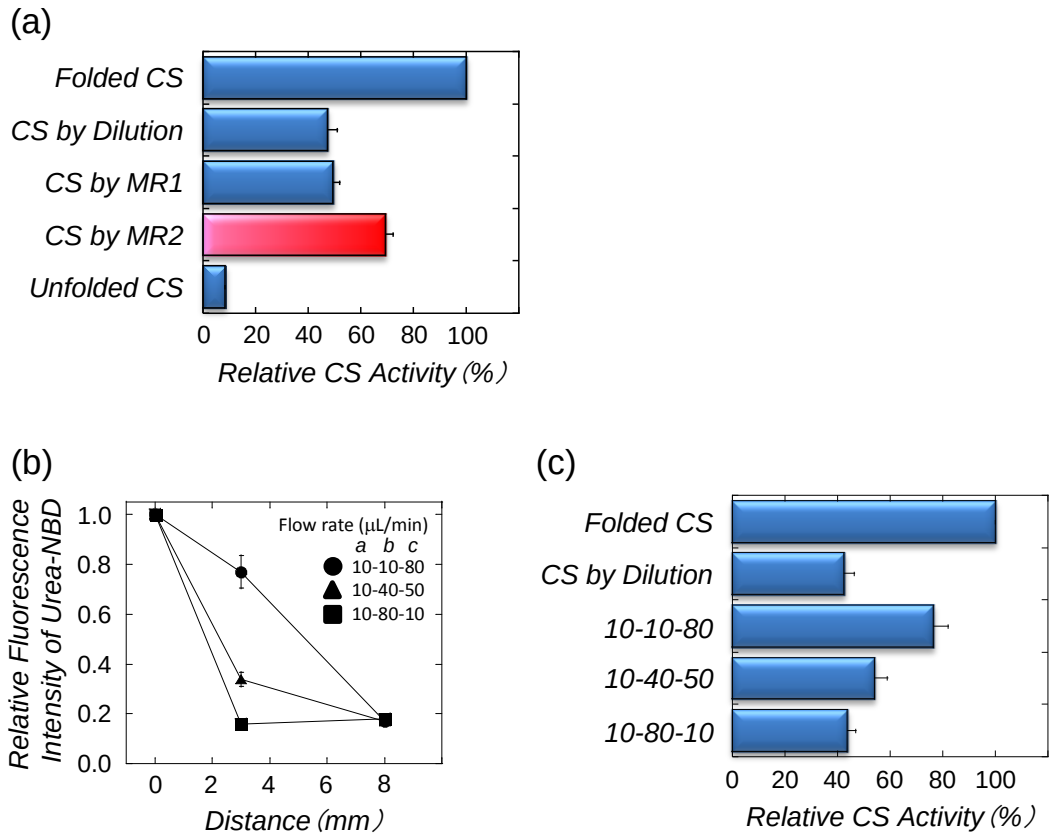
4.2. Protein Refolding Using Microfluidic Chips

To test the performance of the designed microfluidic chips, the refolding of citrate synthase (CS) was tested [15]. CS is known to have low refolding yield using dialysis and dilution [13]. Therefore, CS has been used as a test case for refolding strategies [47,49]. In addition, CS is a dimeric protein composed of two identical subunits, suggesting that CS is a good model protein to study, not only the secondary and tertiary structures, but also the quaternary structure.

The refolded CS prepared using MR1 with one junction showed similar recovered enzymatic activity (<50%) to that of the diluted sample, and less helical structure than the folded CS sample, suggesting that rapid diffusion of urea from the denatured CS leads to misfolding. In contrast, the refolded CS sample

prepared using MR2 with two junctions showed a more helical structure than CS prepared by dilution and MR1 [15]. The recovered activity was also enhanced in CS prepared using MR2 compared with the batch and MR1 samples (Figure 5a). These results indicate that denatured CS was more efficiently refolded using MR2 compared with protein refolding by dilution and MR1. The recovered enzymatic activity of CS by MR2 (>70%) is a similar value to protein refolding by the artificial chaperone-assisted system, which is an efficient technique to recover active proteins from denatured forms [47,48].

Figure 5. Citrate synthase (CS) refolding by microfluidic chips [15]. (**a**) The recovered enzymatic activities of CS using different refolding approaches. Flow rates for MR1: channel *a* (denatured CS), 10 μL/min; and channel *b* (buffer), 90 μL/min. Flow rates for MR2: channel *a* (denatured CS), 10 μL/min; channel *b* (buffer), 10 μL/min; and channel *c* (buffer), 80 μL/min. Folded CS was prepared by dialysis. Unfolded CS was assayed in 2.5 M urea. The diluted sample was directly diluted by buffer in a test tube; (**b**) The effect of the different flow rates of the diluting buffers (channels *b* and *c*) on CS refolding. Relative fluorescence intensities of NBD in the urea stream at the junctions in MR2; (**c**) The recovered enzymatic activities of refolded CS in MR2. Flow rates of channels *a*, *b*, and *c* (μL/min) are shown in the graph.



The laminar flow conditions allowed control of the urea distribution in the microchannel of MR2 (Figure 4c). Thus, the effect of different flow rate ratios of the refolding buffers on the CS refolding yield was studied. In this experiment, the flow rate of denatured CS was maintained at 10 μL/min (channel *a*) and the total flow rate of the refolding buffer was 90 μL/min (channels *b* and *c*) with different flow rate ratios (flow rate in channel *b*:flow rate in channel *c*). Changes of the flow rate ratio of the

buffers showed different decreasing trends of the urea concentrations (Figure 5b). A low flow rate of the buffer in channel *b* (10-10-80 in Figures 4 and 5) resulted in a slow decrease in the urea concentration, leading to highly recovered enzymatic activities (Figure 5c).

The refolding of proteins using microfluidic chips with multiple junctions was achieved within a short period of time at room temperature. The estimated throughput of our CS refolding method, using MR2, was 150 μg/h. This value is one order of magnitude higher than the throughput of the artificial chaperone-assisted system (9–14 μg/h in Ref. 49). These results suggest that a gradual decrease of the denaturant concentration in the microchannel can provide the equilibrium between unfolding and refolding (native conformation) and not misfolding and/or aggregation.

4.3. Refolding of Recombinant Protein from Inclusion Body by Microfluidic Chips

The designed chips have been evaluated for their refolding performance on recombinant protein from inclusion bodies. ζ-Associated protein 70 kDa (ZAP-70) is a tyrosine kinase [64]. Because overexpressed ZAP-70 in the bacterial expression system makes inclusion bodies, it is usually expressed in the mammalian or insect expression system [64,65]. However, these expression systems are expensive compared with the *E. coli* system, and the recovered yield of the protein is generally low.

In the refolding experiments, the urea-denatured ZAP-70 protein kinase domain (mouse residue 337–597), which was purified from *E. coli* inclusion bodies, was applied to the microfluidic chips to evaluate protein refolding. The circular dichroism (CD) spectrum of refolded ZAP-70 using MR1 showed a similar spectrum to the batch sample by dilution. In contrast, the CD spectrum of ZAP-70 prepared using MR2 was similar to that of folded ZAP-70 prepared by step-wise dialysis over two days. The estimated helical content of ZAP-70 using MR2 was higher than that of ZAP-70 prepared using MR1 and dilution [15].

These results indicate that microfluidic chips may provide miniaturized tools for rapid and efficient recovery of active proteins from inclusion bodies.

5. Conclusions

Recovering biologically active proteins at low cost is the important goal in protein refolding from bacterial inclusion bodies, not only for analysis of the protein structure and function [66], but also for the development of therapeutic drugs and industrial processes [1,2]. Refolding is the change of the protein conformation from unfolded to folded, and is dependent on the denaturant concentration. As rapid decreases in denaturant concentration lead to misfolding and/or aggregation [17,19], a gradual decrease in denaturant concentration within a short period of time may lead to efficient protein refolding. In this review, we introduced the refolding methods using laminar flow in microfluidic chips and chemical additives. The estimated throughput of the protein refolding method using microfluidic chips with multiple junctions is 100–200 μg/h [15], suggest that this refolding method may serve as miniaturized tool for laboratory-scale protein recovery. In contrast, although the refolding method using chemical additives may be suitable for industrial purposes, identifying the refolding buffer conditions, such as pH and ionic strength, and the selection of suitable chemical additives is still a major bottleneck. Thus, an automated robotic platform has also been studied to develop a screening system for the dilution refolding process [67].

Recently, an artificial chaperone-assisted refolding method was combined with microfluidic technology [58]. Although protein refolding by dilution in a microchannel led to the formation of protein aggregates because of the rapid removal of denaturant, the artificial chaperone molecule effectively suppressed these protein aggregates at the mixing point of the denatured protein and the diluting buffer [58]. Another combination technique with a molecular chaperone and polyethylene glycol has been reported [68]. These studies suggest that combination of the different technologies is a promising approach and could improve the results of current protein refolding techniques. In addition, studies on the interaction between proteins and between proteins and chemical reagents, such as denaturants and additives, may help us to understand the molecular mechanisms in detail and could progress refolding technology.

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Conflict of Interest

The authors declare no conflict of interest.

References

1. Swartz, J.R. Advances in *Escherichia coli* production of therapeutic proteins. *Curr. Opin. Biotechnol.* **2001**, *12*, 195–201.
2. Clark, E.D.B. Protein refolding for industrial processes. *Curr. Opin. Biotechnol.* **2001**, *12*, 202–207.
3. Baneyx. F. Recombinant protein expression in *Escherichia coli. Curr. Opin. Biotechnol.* **1999**, *10*, 411–421.
4. Baneyx, F.; Mujacic, M. Recombinant protein folding and misfolding in *Escherichia coli*. *Nat. Biotechnol.* **2004**, *22*, 1399–1408.
5. Prasad, S.; Khadatare, P.B.; Roy, I. Effect of chemical chaperones in improving the solubility of recombinant proteins in *Escherichia coli*. *Appl. Environ. Microbiol.* **2011**, *77*, 4603–4609.
6. Li, M.; Su, Z.-G.; Janson, J.-C. *In vitro* protein refolding by chromatographic procedures. *Protein Expr. Purif.* **2004**, *33*, 1–10.
7. Sakono, M.; Kawashima, Y.; Ichinose, H.; Maruyama, T.; Kamiya, N.; Goto, M. Direct refolding of inclusion bodies using reversed micelles. *Biotechnol. Prog.* **2004**, *20*, 1783–1787.
8. Nara, T.Y.; Togashi, H.; Sekikawa, C.; Kawakami, M.; Yaginuma, N.; Sakaguchi, K.; Mizukami, F.; Tsunoda, T. Use of zeolite to refold a disulfide-bonded protein. *Colloids Surf. B Biointerfaces*. **2009**, *68*, 68–73.
9. Zhi, W.; Landry, S.J.; Gierasch, L.M.; Srere, P.A. Renaturation of citrate synthase: Influence of denaturant and folding assistants. *Protein Sci.* **1992**, *1*, 522–529.
10. Eiberle, M.K.; Jungbauer, A. Technical refolding of proteins: Do we have freedom to operate? *Biotechnol. J*. **2010**, *5*, 547–559.

11. Gautam, S.; Dubey, P.; Rather, G.M.; Gupta, M.N. Non-chromatographic strategies for protein refolding. *Recent Pat. Biotechnol.* **2012**, *6*, 57–68.
12. Machold, C.; Schlegl, R.; Buchinger, W.; Jungbauer, A. Matrix assisted refolding of proteins by ion exchange chromatography. *J. Biotechnol.* **2005**, *117*, 83–97.
13. Zhi, W.; Landry, S.J.; Gierasch L.M.; Srere, P.A. Renaturation of citrate synthase: Influence of denaturant and folding assistants. *Protein Sci.* **1992**, *1*, 522–529.
14. Reddy, K.R.C.; Lilie, H.; Rudolph, R.; Lange, C. L-Arginine increases the solubility of unfolded species of hen egg white lysozyme. *Protein Sci.* **2005**, *14*, 929–935.
15. Yamaguchi, H.; Miyazaki, M.; Briones-Nagata, M.P.; Maeda. H. Refolding of difficult-to-fold proteins by a gradual decrease of denaturant using microfluidic chips. *J. Biochem.* **2010**, *147*, 895–903.
16. Marston, F.A.O. The purification of eukaryotic polypeptides synthesized in *Escherichia coli*. *Biochem. J.* **1986**, *240*, 1–12.
17. Tsumoto, K.; Ejima, D.; Kumagai I.; Arakawa, T. Practical considerations in refolding proteins from inclusion bodies. *Protein Expr. Purif.* **2003**, *28*, 1–8.
18. Umetsu, M.; Tsumoto, K.; Hara, M.; Ashish, K.; Goda, S.; Adschiri, T.; Kumagai, I. How additives influence the refolding of immunoglobulin-folded proteins in a stepwise dialysis system. Spectroscopic evidence for highly efficient refolding of a single-chain Fv fragment. *J. Biol. Chem.* **2003**, *278*, 8979–8987.
19. Ho, J.G.S.; Middelberg, A.P.J.; Ramage P.; Kocher, H.P. The likelihood of aggregation during protein renaturation can be assessed using the second virial coefficient. *Protein Sci.* **2003**, *12*, 708–716.
20. Liu, W.; Cellmer, T.; Keerl, D.; Prausnitz, J.M.; Blanch, H.W. Interactions of lysozyme in guanidinium chloride solutions from static and dynamic light-scattering measurements. *Biotchnol. Bioeng.* **2005**, *90*, 482–490.
21. Hevehan, D.L.; Clark, E.D.B. Oxidative renaturation of lysozyme at high concentrations. *Biotechnol. Bioeng.* **1997**, *54*, 221–230.
22. Orsini, G.; Goldberg, M.E. The renaturation of reduced chymotrypsinogen A in guanidine HCl. Refolding *versus* aggregation. *J. Biol. Chem.* **1978**, *253*, 3453–3458.
23. Yamaguchi, S.; Yamamoto, E.; Mannen, T.; Nagamune T. Protein refolding using chemical refolding additives. *Biotechnol. J.* **2013**, *8*, 17–31.
24. Kudou, M.; Yumioka, R.; Ejima, D.; Arakawa, T.; Tsumoto, K. A novel protein refolding system using lauroyl-L-glutamate as a solubilizing detergent and arginine as a folding assisting agent. *Protein Expr. Purif.* **2011**, *75*, 46–54.
25. Ohtake, S.; Kita, Y.; Arakawa, T. Interactions of formulation excipients with proteins in solution and in the dried state. *Adv. Drug Deliv. Rev.* **2011**, *63*, 1053–1073.
26. Lin, W.J.; Traugh, J.A. Renaturation of casein kinase II from recombinant subunits produced in *Escherichia coli*: Purification and characterization of the reconstituted holoenzyme. *Protein Expr. Purif.* **1993**, *3*, 256–264.
27. Arora, D.; Khanna, N. Method for increasing the yield of properly folded recombinant human gamma interferon from inclusion bodies. *J. Biotechnol.* **1996**, *52*, 127–133.

28. Bell, S.; Hansen, S.; Buchner, J. Refolding and structural characterization of the human p53 tumor suppressor protein. *Biophys. Chem.* **2002**, *96*, 243–257.
29. Asano, R.; Kudo, T.; Makabe, K.; Tsumoto, K.; Kumagai, I. Antitumor activity of interleukin-21 prepared by novel refolding procedure from inclusion bodies expressed in *Escherichia coli*. *FEBS Lett.* **2002**, *528*, 70–76.
30. Mande, S.C.; Sobhia, M.E. Structural characterization of protein-denaturant interactions: Crystal structures of hen egg-white lysozyme in complex with DMSO and guanidinium chloride. *Protein Eng.* **2000**, *13*, 133–141.
31. Ito, L.; Shiraki, K.; Matsuura, T.; Okumura, M.; Hasegawa, K.; Baba, S.; Yamaguchi, H.; Kumasaka, T. High-resolution X-ray analysis reveals binding of arginine to aromatic residues of lysozyme surface: Implication of suppression of protein aggregation by arginine. *Protein. Eng. Des. Sel.* **2011**, *24*, 269–274.
32. Matsuoka, T.; Hamada, H.; Matsumoto, K.; Shiraki, K. Indispensable structure of solution additives to prevent inactivation of lysozyme for heating and refolding. *Biotechnol. Prog.* **2009**, *25*, 1515–1524.
33. Hamada, H.; Shiraki, K. L-Argininamide improves the refolding more effectively than L-arginine. *J. Biotechnol.* **2007**, *130*, 153–160.
34. Ito, L.; Shiraki, K.; Makino, M.; Hasegawa, K.; Kumasaka, T. Glycine amide shielding on the aromatic surfaces of lysozyme: Implication for suppression of protein aggregation. *FEBS Lett.* **2011**, *585*, 555–560.
35. Samuel, D.; Kumar, T.K.; Ganesh, G.; Jayaraman, G.; Yang, P.W.; Chang, M.M.; Trivedi, V.D.; Wang, S.L.; Hwang, K.C.; Chang, D.K.; Yu, C. Proline inhibits aggregation during protein refolding. *Protein Sci.* **2000**, *9*, 344–352.
36. Timasheff, S.N. Protein hydration, thermodynamic binding, and preferential hydration. *Biochemistry* **2002**, *41*, 13473–13482.
37. Kohyama, K.; Matsumoto, T.; Imoto, T. Refolding of an unstable lysozyme by gradient removal of a solubilizer and gradient addition of a stabilizer. *J. Biochem.* **2010**, *147*, 427–431.
38. Nian, R.; Kim, D.S.; Tan, L.; Kim, C.W.; Choe, W.S. Synergistic coordination of polyethylene glycol with ClpB/DnaKJE bichaperone for refolding of heat-denatured malate dehydrogenase. *Biotechnol. Prog.* **2009**, *25*, 1078–1085.
39. Webb, S.D.; Cleland, J.L.; Carpenter, J.F.; Randolph, T.W. A new mechanism for decreasing aggregation of recombinant human interferon-gamma by a surfactant: Slowed dissolution of lyophilized formulations in a solution containing 0.03% polysorbate 20. *J. Pharm. Sci.* **2002**, *91*, 543–558.
40. Akbari, N.; Khajeh, K.; Ghaemi, N.; Salemi, Z. Efficient refolding of recombinant lipase from Escherichia coli inclusion bodies by response surface methodology. *Protein Expr. Purif.* **2010**, *70*, 254–259.
41. Paul, S.; Punam, S.; Chaudhuri, T.K. Chaperone-assisted refolding of *Escherichia coli* maltodextrin glucosidase. *FEBS J.* **2007**, *274*, 6000–6010.
42. Wang, F.; Liu, Y.; Li, J.; Ma, G.; Su, Z. On-column refolding of consensus interferon at high concentration with guanidine-hydrochloride and polyethylene glycol gradients. *J. Chromatogr. A.* **2006**, *1115*, 72–80.

43. Hart; R.A.; Lester, P.M.; Reifsnyder, D.H.; Ogez, J.R.; Builder, S.E. Large scale, in situ isolation of periplasmic IGF-I from *E. coli*. *Bio/Technol.* **1994**, *12*, 1113–1117.
44. Bajorunaite, E.; Cirkovas, A.; Radzevicius, K.; Larsen, K.L.; Sereikaite, J.; Bumelis, V.A. Anti-aggregatory effect of cyclodextrins in the refolding process of recombinant growth hormones from *Escherichia coli* inclusion bodies. *Int. J. Biol. Macromol.* **2009**, *44*, 428–434.
45. Sharma, L.; Sharma, A. Influence of cyclodextrin ring substituents on folding-related aggregation of bovine carbonic anhydrase. *Eur. J. Biochem.* **2001**, *268*, 2456–2463.
46. Vandevenne, M.; Gaspard, G.; Belgsir, E.M.; Ramnath, M.; Cenatiempo, Y.; Marechal, D.; Dumoulin, M.; Frere, J.M.; Matagne, A.; Galleni, M.; Filee, P. Effects of monopropanediamino-β-cyclodextrin on the denaturation process of the hybrid protein BlaPChBD. *Biochim. Biophys. Acta.* **2011**, *1814*, 1146–1153.
47. Daugherty, D.L.; Rozema, D.; Hanson, P.E.; Gellman, S.H. Artificial chaperone-assisted refolding of citrate synthase. *J. Biol. Chem.***1998**, *273*, 33961–33971.
48. Rozema, D.; Gellman, S.H. Artificial chaperone-assisted refolding of carbonic anhydrase B. *J. Biol. Chem.* **1996**, ***271***, 3478–3487.
49. Machida, S.; Ogawa, S.; Xiaohua, S.; Takaha, T.; Fujii, K.; Hayashi, K. Cycloamylose as an efficient artificial chaperone for protein refolding. *FEBS Lett.* **2000**, *486*, 131–135.
50. Haeberle, S.; Zengerle, R.K. Microfluidic platforms for lab-on-a-chip applications. *Lab Chip* **2007**, *7*, 1094–1110.
51. Ohno, K.; Tachikawa, K.; Manz, A. Microfluidics: Applications for analytical purposes in chemistry and biochemistry. *Electrophoresis* **2008**, *29*, 4443–4453.
52. Asanomi, Y.; Yamaguchi, H.; Miyazaki, M.; Maeda, H. Enzyme-immobilized microfluidic process reactors. *Molecules* **2011**, *16*, 6041–6059.
53. Yamaguchi, H.; Maeki, M.; Yamashita, K.; Nakamura, H.; Miyazaki, M.; Maeda, H. Controlling one protein crystal growth by droplet-based microfluidic system. *J. Biochem.* **2013**, *153*, 339–346.
54. Lapidus, L.J.; Yao, S.; Mcgarrity, K.S.; Hertzog, D.E.; Tubman, E.; Bakajin, O. Protein hydrophobic collapse and early folding steps observed in a microfluidic mixer. *Biophys. J.* **2007**, *93*, 218–224.
55. Kane, A.S.; Hoffmann, A.; Baumgärtel, P.; Seckler, R.; Reichardt, G.; Horsley, D.A.; Schuler, B.; Bakajin, O. Microfluidic mixers for the investigation of rapid protein folding kinetics using synchrotron radiation circular dichroism spectroscopy. *Anal. Chem.* **2008**, *80*, 9534–9541.
56. Hertzog, D.E.; Michalet, X.; Jäger, M.; Kong, X.; Santiago, J.G.; Weiss, S.; Bakajin, O. Femtomole mixer for microsecond kinetic studies of protein folding. *Anal. Chem.* **2004**, *76*, 7169–7178.
57. Kerby, M.B.; Lee, J.; Ziperstein, J.; Tripathi, A. Kinetic measurements of protein conformation in a microchip. *Biotechnol. Prog.* **2006**, *22*, 1416–1425.
58. Yamamoto, E.; Yamaguchi, S.; Sasaki, N.; Kim, H.-B.; Kitamori, T.; Nagamune, T. Artificial chaperone-assisted refolding in a microchannel. *Bioprocess Biosyst. Eng.* **2010**, *33*, 171–177.
59. Karnik, R.; Gu, F.; Basto, P.; Cannizzaro, C.; Dean, L.; Kyei-Manu, W.; Langer, R.; Farokhzad, O.C. Microfluidic platform for controlled synthesis of polymeric nanoparticles. *Nano Lett.* **2008**, *8*, 2906–2912.

60. Hong, S.; Tsou, P.H.; Chou, C.K.; Yamaguchi, H.; Su, C.B.; Hung, M.C.; Kameoka, J. Microfluidic three-dimensional hydrodynamic flow focusing for the rapid protein concentration analysis. *Biomicrofluidics* **2012**, *6*, 24132.
61. Dayel, M.J.; Hom, E.F.; Verkman, A.S. Diffusion of green fluorescent protein in the aqueous-phase lumen of endoplasmic reticulum. *Biophys. J.* **1999**, *76*, 2843–2851.
62. Gosting, L.J.; Akeley, D.F. A study of the diffusion of urea in water at 25 ° with the Gouy interference method. *J. Am. Chem. Soc.* **1952**, *74*, 2058–2060.
63. Jahn, A.; Vreeland, W.N.; deVoe, D.L.; Locascio, L.E.; Gaitan, M. Microfluidic directed formation of liposomes of controlled size. *Langmuir* **2007**, *23*, 6289–6293.
64. Jin, L.; Pluskey, S.; Petrella, E.C.; Cantin, S.M.; Gorga, J.C.; Rynkiewicz, M.J.; Pandey, P.; Strickler, J.E.; Babine, R.E.; Weaver, D.T.; Seidl, K.J. The three-dimensional structure of the ZAP-70 kinase domain in complex with staurosporine. *J. Biol. Chem.* **2004**, *279*, 42818–42825.
65. Visco, C.; Magistrelli, G.; Bosotti, R.; Perego, R.; Rusconi, L.; Toma, S.; Zamai, M.; Acuto O.; Isacchi, A. Activation of Zap-70 tyrosine kinase due to a structural rearrangement induced by tyrosine phosphorylation and/or ITAM binding. *Biochemistry* **2000**, *39*, 2784–2791.
66. Swietnicki, W. Folding aggregated proteins into functionally active forms. *Curr. Opin. Biotechnol.* **2006**, *17*, 367–372.
67. Ordidge, G.C.; Mannall, G.; Liddell, J.; Dalby, P.A.; Micheletti, M. A generic hierarchical screening method for the analysis of microscale refolds using an automated robotic platform. *Biotechnol. Prog.* **2012**, *28*, 435-444.
68. Nian, R.; Kim, D.S.; Tan, L.; Kim, C.W.; Choe, W.S. Synergistic coordination of polyethylene glycol with ClpB/DnaKJE bichaperone for refolding of heat-denatured malate dehydrogenase. *Biotechnol. Prog.* **2009**, *25*, 1078–1085.