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Technical refolding of proteins: Do we have freedom to operate?

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11 **Technical Refolding of Proteins, Do we have Freedom to**
12 **Operate?**
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Abstract

Expression as inclusion bodies in *Escherichia coli* is a widely used method for the large-scale production of therapeutic proteins, that do not require post-translational modifications. High expression yields and simple recovery steps of inclusion bodies from the host cells are attractive features in the industrial scale. However, the value of an inclusion body based process is dominated by the solubilization and refolding technologies. Scale-invariant technologies, economically and applicable for a wide range of proteins are requested by industry. The main challenge is to convert the denatured protein in its native conformation at high yields. Refolding competes with misfolding and aggregation. Thus, yield of native monomer depends strongly on the initial protein concentrations in the refolding solution. Reasonable yields are attained at low concentrations (≤ 0.1 mg/mL). However, that requires large buffer tanks and time-consuming concentration steps. We attempt to give an answer to which extent refolding of proteins is protected by patents. Low-molecular mass additives have been developed to improve refolding yields through the stabilization of the protein in the solution and shielding hydrophobic patches. Progresses were established in the field of high-pressure renaturation and on-column refolding. Mixing times of the denatured protein in the refolding buffer were reduced by newly developed devices and the introduction of specific mixers. Concepts of continuous refolding were introduced in order to reduce tank sizes and increase yields. A few patents covering refolding of proteins will expire soon or have expired. This gives more freedom to operate.

Keywords: Inclusion bodies, refolding, *E. coli*, recombinant proteins, on column, additives

Introduction

Approximately 40 % of all biopharmaceuticals are produced in *E. coli* cells [1]. *E. coli* cells grow rapidly to high cell densities on inexpensive substrates and well established fermentation strategies are attractive for an economic expression in industrial scales. Furthermore, the genetic properties of *E. coli* are well characterized and the strains are easy to handle. This explains why *E. coli* is an economic and efficient production system, and widely used for the expression of recombinant proteins [2,3]. However, recombinant proteins are not always folded in their proper and active conformation during protein biosynthesis. For a broad majority of heterologous proteins, secretion in *E. coli* results in 0.5 to 0.8 g L⁻¹ volumetric yield [4]. Higher titers can be attained, but usually require an extensive engineering of the fermentation protocol and expression system [5,6,7]. Thus many products on the market are produced as inclusion bodies in the cytoplasm of *E. coli*, where high fermentation titers can be achieved according to Biopharmaceutical Products in the US and European Markets 6th edition. Inclusion bodies contain the target protein as insoluble aggregates, present in a kind of paracrystalline form. The proteins exist in non-native conformations but with a certain content of secondary structure elements [8]. After solubilization with chaotropic buffers in a reducing environment an elaborative renaturation step is required to refold the protein in its native and active conformation [9]. Anyway, this technology is widely used. Beside high expression yields there are other benefits, that compensate the disadvantage of an additional refolding step: Inclusion bodies have a higher density (~1.3 g/mL) than other cellular components and cell debris and can be easily separated and purified by a combination of cell homogenization and centrifugation. During expression the target protein accumulates in the inclusion bodies and is mostly resistant to proteolytic attacks of cell proteases. After primary isolation of inclusion bodies adhesive impurities such as cell debris and host cell proteins can be reduced by several wash and centrifugation steps. Finally a high purity of up to 90 % of the product protein can be achieved in inclusion bodies. This simplifies and reduces subsequent purification steps [10,11].

The process for the production of recombinant protein from inclusion bodies comprises cell cultivation and harvest, disruption, recovery of inclusion bodies, solubilization, refolding and reoxidation of disulphide bonds and further purification steps (Figure 1) [11,12]. If inclusion bodies contain high amounts of impurities, a denatured purification step of the solubilized protein can be performed prior to refolding. Popular methods are ion exchange, size exclusion or metal ion affinity chromatography [13,14,15].

1 Inclusion bodies are aggregated and densely packed paracrystalline forms of protein. These
2 refractile particles are solubilized in high concentrations of chaotropic agents such as urea or
3 guanidine hydrochloride. Reducing conditions are inevitable, as non-native intra- and
4 intermolecular disulfide bonds may have been formed in inclusion bodies during translation.
5 Solubilization results in a protein in its denatured form. The subsequent step transfers the
6 unfolded and reduced protein into conditions, where the formation of native and bioactive
7 structures is favored. Of all process steps, refolding is the most crucial step. It decides on the
8 efficiency of an inclusion body based process [16,17,18].

9 Renaturation is initiated by reducing or removing the chaotropic solvent. The yield of the
10 refolding step depends strongly on the renaturation conditions such as pH, redox conditions,
11 buffer additives and protein concentrations. These conditions have been empirically optimized
12 for each individual protein.

13 Most proteins contain cysteine residues that form disulfide bonds, which are required for the
14 formation of a native, bioactive structure. For *in vitro* refolding it is usually essential to add a
15 redox buffer system to support the formation of native disulfide bonds. Supplementing the
16 refolding buffer with low molecular weight thiol reagents allows the formation of disulfide
17 bonds, as well as the reshuffling of incorrect formed bonds. Generally a combination of a
18 reduced and oxidized component is used, for example cysteine/cystine or reduced/oxidized
19 glutathione. Suitable ratios must be found to maximize yields [16,19]. Molar ratios of reduced
20 to oxidized agents are recommended between 5:1 and 1:1. These ratios provide a suitable
21 redox potential for the formation and reshuffling of disulfide bonds [20,21].

22 However, the correct folding pathway competes often in disadvantage, with aggregation and
23 misfolding of the target protein. These two reactions dominate the efficiency of the *in vitro*
24 refolding step. After dilution of the unfolded and reduced protein in a refolding buffer,
25 transient intermediates (I) are formed (Figure 2). Usually, these intermediates are partially
26 folded and hydrophobic patches are not completely buried in the core of the protein. There are
27 two proposed reaction pathways for these intermediates. One leads to the native
28 conformation, where intramolecular interactions are involved. This reaction follows a kinetics
29 of first order. The other pathway directs to aggregates where intermolecular interactions are
30 responsible for a second- or higher-order reaction. This kinetic partitioning explains
31 decreasing refolding yields with increasing initial concentrations of unfolded and reduced
32 protein [22,23,24,25]. The decline of the refolding yield with rising concentrations of the
33 denatured protein is demonstrated in Figure 3 for α -lactalbumin and a single-chain antibody
34 fragment (scFv). The susceptibility of aggregation is here clearly higher for the scFv. At
35 concentrations ≤ 0.1 mg/mL both proteins can be totally renatured. However, increasing the
36 concentration of the denatured protein leads to a tremendous loss of native protein. As

1 aggregation results from nonspecific interactions between hydrophobic regions or partially
2 folded structures of different polypeptide chains, it is usually irreversible [26]. Suppressing
3 aggregation is therefore an inevitable step to achieve high yields of native and active protein.
4 A common process for protein refolding in industry is batch dilution at very low protein
5 concentrations (≤ 0.1 mg/mL) [20,27]. However, this requires large volumes of refolding
6 buffer, huge reactors and additional concentration steps. Beside high costs for time-
7 consuming steps, waste disposal has also to be taken into account. Therefore the refolding
8 step needs to be carefully optimized, to gain a simple and cost-effective bioprocess [16]. As
9 consequence, conditions have to be found, where the hydrophobic interactions are suppressed
10 and refolding to the native protein is favored. Unwanted side reactions as misfolding and
11 aggregation have to be decelerated. Thus a lot of research and development have been done to
12 optimize various parameters in the refolding step. One approach is the search and
13 development of new additives, supporting the solubility after dilution and shield hydrophobic
14 patches during folding. Other approaches focus on the addition mode of the denatured protein
15 to the renaturation buffer. Reduced mixing times result in lower local protein concentrations
16 and decrease aggregation rates [10]. Refolding under high pressures or on a column matrix is
17 also a possibility to attain high recovery rates in a renaturation process [9,10].

18 It is not the purpose of the manuscript to give advice how to circumvent existing patents. We
19 want to draw attention to patent literature, which may prevent the application of certain
20 refolding procedures. Mainly we focus on process patents, less on patents expressing a certain
21 protein a unique refolding conditions.

22 **Refolding conditions and processes**

23 *Refolding by direct dilution*

24 The simplest method to refold proteins is the addition of the unfolded and reduced protein
25 directly to the refolding buffer. In biopharmaceutical manufacturing this is a widely used
26 method, as this addition mode is cheap and easy to scale and validate, respectively. Due to the
27 aggregation characteristics of the proteins, concentrations are kept low (usually ≤ 0.1 mg/mL)
28 to attain reasonable refolding yields. Higher concentrations enhance the probability of
29 collisions of unfolded or partially folded protein and lead to higher aggregation rates. Thus
30 working at higher concentrations demand a controlled addition of the denatured protein to the
31 renaturation buffer. Aggregation is reduced, if low local protein concentrations are rapidly
32 achieved in the refolding tank. Therefore, short mixing times are inevitable for an efficient
33 process. To increase the yield of native protein, dilution can be done as fed-batch and
34 continuous dilution. Several groups have claimed these methods but patent protection have
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1 been recently explored [28,29]. In a stirred tank various parameters influence the refolding
2 yield, as the intensity of mixing, the injection rate, the injection point, the concentration of
3 denatured protein and the total protein concentration [30,31]. For example, lysozyme shows
4 higher refolding yields in fed-batch addition compared to simple batch dilution [32,33].
5
6 Another continuous solution was filed by Buus et al. in 2002 [34]. In a mixing chamber the
7 denatured protein is mixed with the refolding buffer and the refolded protein is recovered
8 through a connected expanded bed absorption column. A benefit is here the possibility to
9 recycle the refolding buffer [35]. As mentioned, the intensity of mixing affects the refolding
10 yield. To avoid aggregation in the tank, mixing times must be shorter than the reaction rate of
11 aggregation. However, in industrial scales large tank reactors exhibit low mixing efficiencies.
12 Efforts to improve mixing in large scale are therefore inevitable [31]. A scaleable, efficient
13 mixing device is an oscillatory flow reactor, invented by Middelberg et al. [36]. The mixing
14 chamber is a column, that contains the refolding buffer. It is divided in eight sections by seven
15 flat ring baffles. The fluid is oscillated through a piston at the bottom of the column. Mixing
16 is controllable through the frequency and the amplitude of the piston. Unfolded protein can be
17 fed continuously into the column, either at one or two feed points. Compared to a standard
18 fed-batch mode in a stirred tank, refolding yields could be almost doubled by this approach
19 [37]. Another possibility to control the intensity of mixing is to involve a static mixing device
20 which was protected by patents of St. John et al. [38] and Schlegl [39]. Here, the refolding
21 buffer is pumped through a static mixer. The concentrated denatured protein is added to the
22 conduit via the inlet upstream of the mixing device. This system achieves at least as good
23 yields as conventional methods. It has the advantages of scalability, higher throughput and
24 robustness of the process. Schlegl extended the approach of a static mixer through the
25 recirculation of the renaturation buffer (Figure 4). Mixing times can be adjusted by the feed
26 time of the denatured and reduced protein solution and by the recirculation flow. A fast
27 collapse of the protein is assumed immediately after folding is initiated. The resulting
28 conformation contains native-like secondary structures and is called the “molten globule”
29 intermediate. These intermediates are often stable under non-denaturing conditions [40].
30 Through the use of a plug flow reactor prior to the refolding tank, the residence time can be
31 adjusted, necessary for the formation of a stable intermediate. This stabilized conformation is
32 less prone to aggregation while the buffer recirculates and further denatured protein is added.
33 Another refolding method is reversed dilution. Most dilution methods feed the denatured
34 protein solution to the refolding buffer. In reversed dilution the concentration of the
35 denaturant as well as of the protein is decreased simultaneously, as the refolding buffer is fed
36 to the solubilized inclusion bodies. He et al. used this approach to refold a staphylokinase
37 variant [41].

1 Current patent literature suggests that fed-batch and simple dilution is not covered by patents,
2 but for several mixing devices patents have been filed.
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5 *Solubilization of inclusion bodies*

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7 Solubilization and refolding are often interrelated. The solubilization agent influences the
8 refolding conditions. Earlier studies on the structure of inclusion bodies disclosed native or
9 native-like structures coexisting with β -forms [42,43]. If the inclusion bodies can be
10 solubilized under conditions where these structures can be conserved, refolding may results in
11 clearly higher yields. Conventional solubilization agents are high concentrations of chaotropes
12 like urea or guanidine hydrochloride that denature the target protein completely. Thus gentle
13 solubilization methods are necessary to keep the secondary native structures that occur in the
14 inclusion bodies. For example, McCoy patented a solubilization process for somatotropin
15 inclusion bodies. These inclusion bodies are soluble in low concentrations of urea (around 2
16 M) at a pH of 12 [44]. Another uncommon solubilization approach was filed by Yong-Jun et
17 al. [45]. They used organic solvents such as n-propyl alcohol or isopropyl alcohol including
18 0.05 % β -mercaptoethanol at a pH around 12 and temperatures of 30 °C and higher.
19 Surfactants as N-lauroyl-sarcosine or sodium dodecyl sulfate (SDS) are also used as
20 solubilizers that do not disrupt existing structures. However, the use of surfactants is usually
21 avoided as it requires extensive purification steps for the removal from the protein solution
22 [17,46]. A total recovery of the target protein from the inclusion bodies is a fundamental goal
23 in the overall process. Using gentle solubilization methods may result in low recovery yields.
24 Additionally misfolded conformations and multimers in the inclusion bodies can not be
25 dissolved and precipitate immediately after refolding is initiated [47]. Aggregation of proteins
26 can be suppressed if definite pH ranges are chosen for solubilization and refolding. At a pH
27 far away from the isoelectric point (pI) a protein is highly charged. Charge repulsion occurs
28 and prevents aggregation [48]. Denaturing and refolding at alkaline pH and subsequent slow
29 reduction of the pH near the pI can support renaturation and was invented by Xinli [49].
30 Pizarro et al. filed a patent for a process that also focuses on the pH in the solubilization and
31 renaturation process [50]. Inclusion bodies were solubilized at pHs greater than 9 and
32 refolding was also accomplished at strong alkaline conditions (pHs between 9 and 11).
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34 It seems that protein solubilization with chaotropic agents is not covered by patents and we
35 have a freedom to operate. When it comes to solubilization a high pH the filed application of
36 Pizarro et al. must be taken into consideration
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Refolding buffer composition: Additives and folding aids

Direct dilution of the denatured and reduced protein in the refolding buffer is a simple and fast method. However, renaturation steps require a carefully optimized buffer composition to attain reasonable yields. Appropriate additives play here a decisive role. They have to meet following requirements: (1) Improve clearly the refolding yield, (2) preserve the cost-effectiveness of the overall process, (3) do not disturb subsequent purification steps and (4) are removable from the final product during the purification process.

Hydrophobic interactions and hydrogen bonds are responsible for the aggregation of proteins. Primarily, unfolded proteins and folding intermediates are prone to aggregation. The addition of low molecular additives or detergents to the refolding buffer reduces aggregation and in consequence precipitation during the renaturation process. Usually, these compounds can be easily removed after the refolding step. Likely, they support the solubility and stability of the native, denatured and intermediate states [51]. The most frequently used additive is L-arginine and it seems that the addition of this compound for enhancing solubility is not protected by a patent. Usually, it is added to the refolding buffer in concentrations of 0.4 to 0.8 M [52,53]. Liu et al. [54] reported, that L-arginine temporarily stabilizes the denatured proteins as well as early partially folded intermediates and refolded protein. Through specific interactions L-arginine slows down conformational movement and consequently protein-protein association during the refolding process [55]. L-arginine as folding additive, especially in industrial scale, is very costly. Chaotropic agents as guanidine hydrochloride and urea can also be used as refolding additives. This, however, requires denaturant concentrations that do not destabilize the native state of the protein [51]. One explanation of the mode of action of urea and guanidine hydrochloride as protein stabilizing agents is the preferential interaction theory. It is considered, that protein stabilizing factors are based on direct protein-denaturant interactions [56]. Pike and Acharya investigated the interactions between urea and lysozyme [57]. Subtle conformational changes took place within the crystal structure of lysozyme upon exposure to urea. These changes were observed at regions of the surface of the molecule, that are known to be relatively flexible. Previous studies indicated that urea and guanidine hydrochloride, present in non-denaturing concentrations, interact with the protein through multiple hydrogen bonds [56,57,58].

Shiraki et al. [59] invented a new refolding additive which is a derivative of arginine. They proofed, that L-argininamide is a better refolding additive compared to the widely used L-arginine [60]. For lysozyme the refolding yield was 1.7-fold higher in the presence of argininamide than for arginine. In the case of bovine carbonic anhydrase the improvement of the final refolding yield was 1.4-fold.

1 A new additive was invented by Flowers and Summers [61]. Hen egg white lysozyme could
2 be successfully refolded at high protein concentrations using ethylammonium nitrate (EAN).
3 Examinations of the effect of EAN on the thermal properties of the enzyme showed, that EAN
4 acts as denaturant. Proteins can be denatured with EAN and refolded by simple dilution,
5 resulting in reasonable refolding yields at high protein concentrations. It is supposed, that the
6 ethyl residue of EAN interacts with the hydrophobic patches and suppresses aggregation. The
7 charged ammonium group is assumed to stabilize the secondary structure through electrostatic
8 interactions. EAN has the main advantage of being easily removed by desalting methods [62].
9 A similar approach was patented by Peters et al. [63]. They presented that chemical denatured
10 protein can be successfully refolded in presence of secondary and tertiary amines. Adding
11 triethanolamine- H_2SO_4 in concentrations around 1 M showed a good refolding efficiency for
12 recombinant interleukin-4. For bovine pancreatic trypsin Tris(hydroxymethyl)-aminoethane
13 (TRIS) in combination with H_2SO_4 appeared as suitable agent for renaturation.

14 Fluorine compounds are also potential renaturants, as invented by Lohr et al. [64]. They could
15 renature human serum albumin after heat denaturation by adding trifluoroethanol to the
16 solution. Examinations with different fluorine derivatives indicated, that compounds with a
17 trifluoromethyl group and a vicinal hydroxyl group are most effective for refolding.

18 Other additives, used in protein folding technology are cyclodextrins. These cyclic glucose
19 oligosaccharides are able to prevent aggregation through non-covalent inclusion complexes
20 with the hydrophobic patches of aggregation prone intermediates. Patents are filed, using this
21 technology [65,66]. Refolding of bovine carbonic anhydrase and recombinant endostatin
22 showed much higher yields, if α -cyclodextrin was added to the renaturation buffer [67].
23 Cyclodextrins are also applied in the approach "artificial chaperoning". This concept was
24 introduced by Rozema et al. [68] in 1995 and is similar to the function of the natural
25 molecular chaperones. It comprises two steps: In the first step detergent molecules are added
26 to capture the non-native species of the protein by forming a protein-detergent complex.
27 Aggregation and also native refolding is disabled. To start renaturation β -cyclodextrin is
28 added in the second step. It strips off the detergent from the protein-detergent complexes
29 and allows the protein to form its native conformation. There are three major cyclodextrins
30 with six, seven and eight glucose units (α -, β -, γ -cyclodextrin, respectively). Cycloamylose
31 has more glucose units, usually between 17 and hundred. Machida et al. [69,70] invented the
32 use of cycloamylose as additive in refolding experiments. In combination with dedicated
33 detergents superior refolding yields could be achieved in comparison to the corresponding
34 conventional α -, β - and γ -cyclodextrins.

35 Another stripping agent was reported by Khodaghali et al. [71]. They used alginate instead of
36 cyclodextrins. Alginate is a linear polysaccharide that consists of β -D-mannuronic acid and α -

1 L-guluronic acid. Electrostatic forces provoke the interaction of the alginate with oppositely
2 charged detergents [72]. Refolding of alkaline phosphatase in dodecyl trimethylammonium
3 bromide (DTAB) and subsequent stripping of the charged detergent with alginate resulted in
4 high recovery yields of native protein [71].
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8 Reversed micelles are also an option for the renaturation of protein derived from inclusion
9 bodies. They are formed, when mixtures of surfactants, water and nonpolar solvents are
10 combined in defined concentrations. The surfactant aerosol OT (AOT, sodium dioctyl
11 sulfosuccinate) is very common for this system. Aqueous nanoscaled droplets are formed,
12 stabilized by surfactants. Through micelles, protein molecules are separated of each other.
13 Intermolecular interactions are reduced and aggregation is prevented. The polar groups are
14 concentrated inside, whereas the lipophilic groups extend to the non-polar organic solvent.
15 The size of the aqueous droplet inside of the micelle depends on the conditions of the system.
16 Variables as surfactant to water ratio, ionic strength, surfactant and protein concentration have
17 to be varied until the size of a micelle conforms to the size of a protein molecule. The total
18 process comprises four steps: (1) The denatured protein has to be transferred into the micelles,
19 (2) the denaturant has to be removed, (3) folding agents as redox reagents can be added and
20 (4) extraction of the renatured protein from the micelles. RNase A was successfully refolded
21 with this system [73,74]. Compared to the conventional dilution method Sakono et al. [73]
22 could improve refolding yields from 40 to 100 %. But the refolding kinetics was slower with
23 the reversed micelle mediated system. To overcome this problem, Sakono et al. added the
24 molecular chaperone GroEL into the reversed micelles. The kinetic was significantly
25 improved. After one hour more than 80% activity was achieved, whereas without GroEL only
26 20 % could be recovered.
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42 Recently, new refolding additives were published by Pitner et al. [75]. They used ionic liquids
43 with a specific distribution of the electron density for protein refolding. Ionic liquids or liquid
44 salts consist usually of an organic cation and an inorganic anion. The cation has at least one
45 electron donor region and one positive charged electrostatic region. Pitner et al. refolded
46 several proteins in presence of ionic liquids. They showed the potency of ionic liquids in
47 comparison to conventional additives, as for example L-arginine.
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52 Mostly the exact mechanism of action of refolding additives is a supposition or even unclear.
53 This makes it inevitable to determine suitable additives as well as refolding conditions for a
54 certain protein by trial and error. It is also not clear, how far the protection of additives
55 reaches. The current practice of patent offices is to strictly limit the invention to the presented
56 example. Thus it is difficult to demonstrate, that a certain additive is suited for all proteins. In
57 particular certain compounds are protected by patents although a general patent on the
58 addition of additives does not exist. An overview of additives is provided in Table 1. We
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1 assume that there are many more compounds have been claimed as additives but often only
2 connection with a certain protein.
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5 *High-pressure disaggregation and folding*

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8 Unfolding of many proteins under high hydrostatic pressure has been known for more than 90
9 years [76]. Pressure is a useful tool to study thermodynamics of proteins, as well as unfolding
10 and refolding processes [77,78]. In general, dissociation of multimeric proteins is facilitated at
11 pressures between 1000 and 3000 bar. For total unfolding pressures up to 8000 bar are usually
12 necessary. The specific volume of a protein state, formed at high pressures, dominate the
13 renaturation process. Simple thermodynamics is responsible for this observation. The pressure
14 dependent change of Gibbs free energy between two states corresponds to their difference in
15 specific volume. Several factors are assumed to cause these volume decreases. Inside folded
16 proteins or at interfaces of oligomeric proteins there are cavities and internal voids, that favor
17 unfolding or dissociation. These intra- and intermolecular cavities are dissected during
18 unfolding processes. Electrostatic interactions are disrupted and the electrostriction of water
19 molecules around free charged groups is caused. Moreover, hydrophobic patches and polar
20 groups are exposed to hydration [79]. High pressure destabilizes native proteins. In
21 magnitude, the occurring change of the specific volume during unfolding is quite small. It is
22 only 0.5 to 1 % of the total specific volume, but nevertheless significant [80].
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25 Pressure induced unfolding is favored as it is reversible and usually only low concentrations
26 or even no chaotropic agents have to be added. Conventional solubilization techniques of
27 inclusion bodies comprise highly concentrated guanidine hydrochloride or urea buffers.
28 Usually proteins are completely unfolded in these chaotropic agents. The combination of high
29 hydrostatic pressure and low concentrations of guanidine hydrochloride prevents aggregation
30 during refolding [81]. Applying high pressure to aggregates or inclusion bodies results in
31 solubilization under gentle conditions. Folding can be started from structures, that are less
32 interrupted or even possess some secondary structure. Studies with RNase A showed, that
33 pressure denatured RNase A keeps a significant degree of secondary structure. Generally it
34 can be stated, that the pressure denatured states of proteins contain more secondary structure
35 than the temperature or chemical denatured states [77].
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38 β -lactamase was successfully refolded from inclusion bodies applying 2000 bar for 48 h, as
39 shown an example of a patent, filed by Randolph et al. [82,83]. Catalytic activity was
40 observed even without a chaotropic agent in the renaturation buffer. At higher concentrations
41 of guanidine the total protein concentration increased and the soluble amount of β -lactamase
42 as well as the recovered activity remained nearly constant. Applying high pressures in the
43 absence of guanidine hydrochloride can be also used as purification step. The total solubilized
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1 protein increased with the concentration of guanidine, whereas the solubilized concentration
2 of the target protein β -lactamase remained the same. As renaturation tool high-pressure has
3 the advantage, that it dissociates aggregates while it favors the native conformation. During
4 refolding it prevents and reverses aggregation, which has already taken place. This technology
5 was invented by Robinson et al. [84]. Additives as urea or guanidine hydrochloride can
6 support this process. That is a major benefit compared to conventional refolding methods, as
7 dilution or dialysis. Aggregation is reversible and higher yields of native protein can be
8 achieved. Even high protein concentrations lead to reasonable refolding yields. As for
9 conventional refolding methods, like dilution, refolding conditions have to be optimized for
10 high pressure renaturation as well. Additives and redox systems have to be tested to improve
11 refolding yields. Using glycerol as folding additive for the enzyme rhodanese in combination
12 with the high-pressure technology nearly doubled the renaturation yield [85]. Other
13 parameters as temperature or agitation during pressure treatment can also promote refolding
14 yields, as patented by Randolph et al. [86]. They showed, that recombinant human growth
15 hormone (rhGH) totally refolded at 60 °C and 2000 bar. Stirring the solution at these
16 conditions was found to increase the refolding yield as well as the folding kinetics. Refolding
17 at high pressures in the presence of specific binding agents was filed by Seefeldt et al. [87].
18 As binding agents small organic molecules, polypeptides and nucleic acids are a possibility
19 for improvements of renaturation yields.

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In the case of biopharmaceutical preparations, aggregates can induce immune responses,
including anaphylactic reactions in patients. The consequences can be fatal. Aggregates do
not only occur during refolding. In processing steps as filtration, ultrafiltration,
chromatography, vial filling, crystallization and so on, protein aggregation can be observed
[88].

The use of high-pressure could be a valuable tool in the preparation and storage of
pharmaceutical drugs to prevent aggregation. In addition, high-pressure technology to
disaggregate and refold proteins is efficient and cost-effective. Refolding at high protein
concentrations does not require large-scale dilution and additional concentration steps. The
plant throughput can be increased which is favorable in industrial production processes.
Currently several patents have been filed to protect high pressure refolding of recombinant
proteins and dissolution of aggregates. In this respect we assume a very limited freedom of
operation and a contact with the owners is advisable.

On-column refolding

There are three different approaches for on-column refolding: (1) Dilution of denaturants by
size exclusion chromatography, (2) retention of the denatured protein on a chromatographic

1 phase and subsequent removal of the denaturant and (3) immobilization of a folding catalyst
2 on a chromatographic phase, where the column acts as a catalyzed refolding reactor [89]. On-
3 column refolding offers the advantage of refolding and simultaneous purification.
4

5 Refolding using size exclusion chromatography is based on a gradual removal of the
6 denaturant. Aggregates, intermediates and native protein are separated by their different
7 diffusion properties in the stationary phase. The denatured protein has a high hydrodynamic
8 radius and is excluded from the gel pores. During refolding the protein size decreases and gel
9 pores are easier accessible. Gu et al. presented, that the overall refolding yields of a single-
10 chain antibody fragment (scFv) with SEC are comparable to yields achieved with batch
11 dilution [90]. Using an urea gradient resulted in slightly higher yields. The highest yield was
12 obtained with a combined urea and pH gradient. However, refolding by SEC has the key
13 advantage over conventional dilution, that the material on the column is fractionated by size.
14 A new method applying SEC for refolding is a continuous process. Intermediates and
15 aggregates are separated from the native refolded protein and can be reconstituted to the feed
16 solution. An extensive study was done with α -lactalbumin as model protein using pressurized
17 continuous annular chromatography (Figure 5). Through recycling of intermediates and
18 aggregates the refolding yield could be improved considerably, which was shown and
19 patented by Necina et al. [91,92].
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22 Adsorption of a denatured protein on a solid matrix is supposed to prevent aggregation
23 through separation of the individual protein molecules from each other during refolding. This
24 approach was firstly invented by Creighton [93]. After adsorption of the unfolded protein on
25 an ion exchange matrix he initiated refolding. As for SEC, P-CAC with an ion-exchange resin
26 achieved better refolding yields for α -lactalbumin, especially when aggregates were
27 reconstituted to the refolding step [79,94]. Another method is dual gradient ion-exchange
28 chromatography (IEC). The denatured protein is eluted by gradual decrease of the denaturant
29 concentration and increase of the pH. The protein refolds gradually and compared to batch
30 dilution higher refolding yields can be attained [95]. Refolding conditions using ion-exchange
31 have to be carefully optimized. Otherwise non-specific protein-matrix interactions can occur
32 and no refolding takes place. However, if optimal conditions are found, denatured protein can
33 be refolded at significantly higher concentrations as in batch dilution [84]. The principle of
34 protein adsorption on an ion exchange matrix can be used in expanded bed adsorption (EBA)
35 chromatography. In EBA chromatography the void fraction is increased which allows the
36 application of particle containing feedstocks. Especially in large scale applications EBA
37 chromatography is a useful tool, has large volumes can be processed. Compared to
38 conventional on-column refolding with an ion exchange resin, similar refolding yields can be
39 attained with EBA chromatography [96].
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1 Simulated moving bed has been used for continuous refolding of proteins [97, 98] but
2 it seems that the method has been never claimed for on-column refolding. Still one might
3 consider that certain configurations or column designs for SMB may be protected by patents.
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6 Introducing a N- or C-terminal polyhistidine-tag allows refolding on a solid matrix
7 based on immobilized metal affinity chromatography (IMAC). Immobilized divalent metal
8 ions (for example Ni^{2+} or Cu^{2+}) form high-affinity complexes with the polyhistidine-tag in
9 presence of high concentrations of denaturants as guanidine or urea. IMAC offers the
10 advantage, that solubilized inclusion bodies are firstly purified and then refolded. Applying
11 IMAC offered superior results for the purification of an anti-TNF α -scFv. A refolding yield of
12 77 % and purity of 95 % could be obtained [99]. The potency of this method was proofed for
13 several proteins, containing a polyhistidine-tag [100,101,102].
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16 Hydrophobic interaction chromatography (HIC) is also an option for on-column refolding.
17 Aggregation can be suppressed through hydrophobic interactions between ligands of the
18 matrix and protein molecules. For lysozyme refolding yields increased with rising
19 hydrophobic strength of the resin. Adding glycerol to the eluent improved the specific activity
20 of the renatured lysozyme [103].
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23 Another set-up for on-column refolding is, to immobilize folding catalysts and
24 artificial chaperones. *In vivo* conditions are mimicked and supposed to improve refolding
25 yields. Tsumoto et al. [104] used the foldase oxidoreductase, immobilized on N-
26 hydroxysuccinimide-activated Sepharose 4FF, to refold a single-chain antibody fragment and
27 prevented the aggregation of the target protein in the refolding step. An engineered chaperone
28 has been also used for improvement of refolding [108,109]. This strategy is not well accepted
29 in industry since it is expensive and a lot of chaperone must be produced because they act in a
30 stoichiometric manner. Patents have been withdrawn and in this respect we also have freedom
31 to operate, presumably somebody finds an inexpensive alternative to produce mini-
32 chaperones.
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35 A novel approach using zeolite was invented by Mizukami et al. [105,106]. Zeolites
36 are crystalline porous aluminosilica compounds. These tectosilicates are made up of AlO_4 and
37 SiO_4 tetrahedra that share the corners. In industry, zeolites are widely used as cationic ion
38 exchangers and catalysts. The main advantage of zeolite is, that proteins can be adsorbed in
39 the presence of denaturants. The zeolite is suspended in the denatured protein solution and
40 after washing away the chaotropic agent, the target protein can be desorbed with buffer
41 containing detergents as polyethylene glycol (PEG) and Tween 20.
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44 On-column refolding provides a good alternative to common methods [107] especially
45 when the refolding yield in batch refolding is very low. The Creighton patent [92] covering a
46 wide range on column refolding methods has a priority date of April 1st 1986 and is
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1 meanwhile expired. So we assume freedom of operation for a conventional on column
2 refolding passing a denatured protein solution over a chromatography column and elution by a
3 kosmotropic buffer. Tough several more sophisticated including continuous chromatographic
4 methods have been filed (Table 1) a general continuous chromatography refolding procedure
5 has not been patented. In particular continuous annular chromatography [92] for continuous
6 refolding has been claimed.
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11 **Conclusion**

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16 *E. coli* is a widely used expression system for the heterologous proteins. The detailed
17 knowledge of the genetics, simple cultivation conditions and short generation times are
18 attractive in commercial and research applications. However, overexpression leads mostly to
19 the accumulation of insoluble inclusion bodies in the cytoplasm of *E. coli* and recovery of
20 native protein requires an elaborate renaturation step. Thus, it is desirable to establish
21 refolding methods, that are applicable for a wide range of different proteins. New approaches
22 and methods have to be investigated to explore their potential as “generic” refolding tools. For
23 manufacturing scale novel concepts have to be evaluated regarding their scalability and
24 economic features. In general, batch and fed batch refolding methods are not covered by
25 patents. The use of improved solubilization and refolding additives is broadly covered, and it
26 is advisable, to check patent literature to avoid infringement. A similar situation has been
27 found for continuous refolding. The freedom to operate is given for technical refolding. There
28 is enough room, to exploit the power of inclusion body technology for further innovative
29 protein products.
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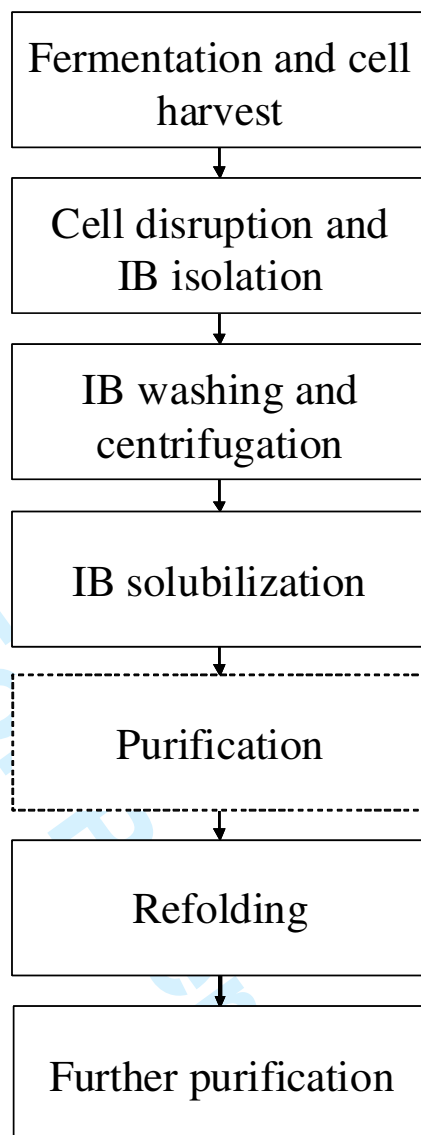


Figure 1: Flow diagram for the protein recovery expressed as inclusion bodies in *E. coli*. – IB: Inclusion bodies.

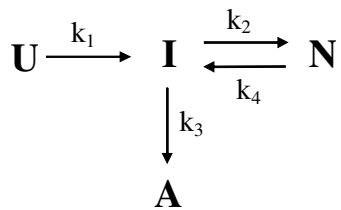


Figure 2: Simplified refolding pathway of a denatured-reduced protein. – U: unfolded and reduced protein, I: intermediate state, N: native state, A: aggregated state, k: reaction rate constant.

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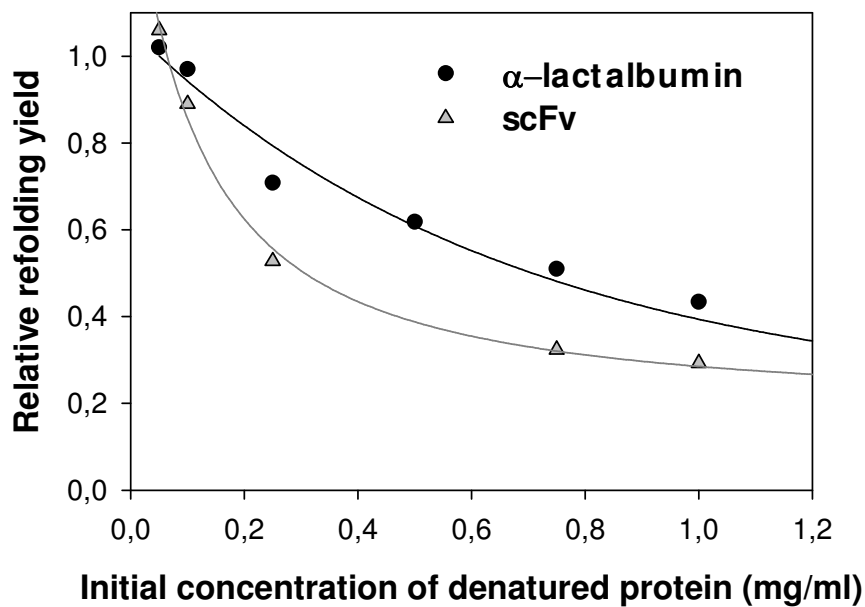
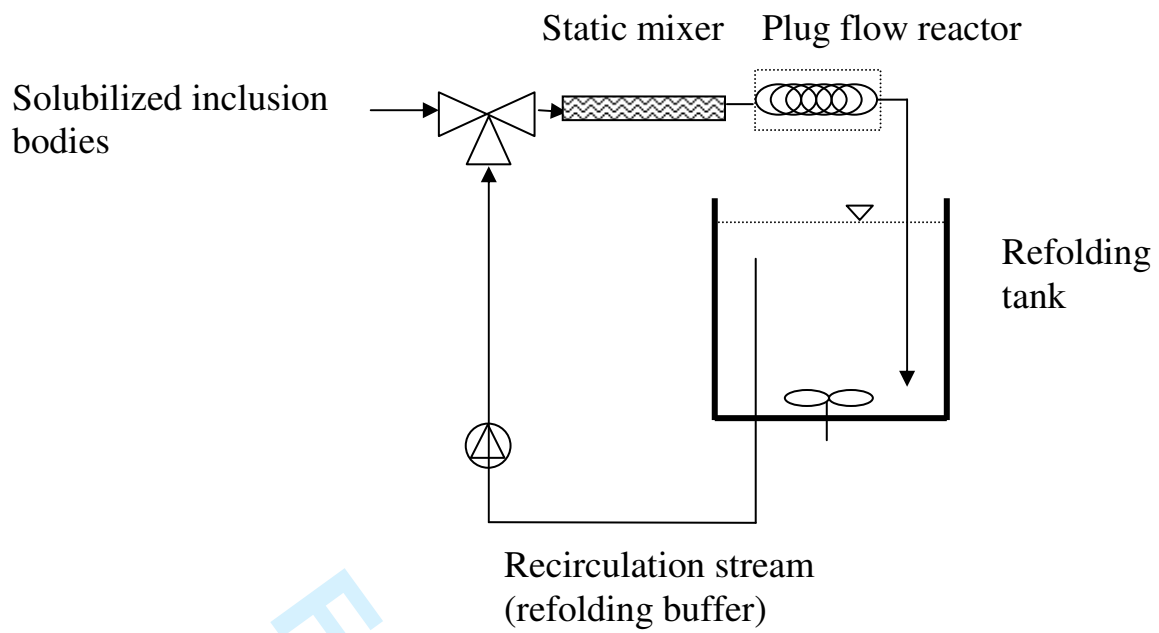


Figure 3: Relative refolding yield dependent on the initial concentration of denatured protein for α -lactalbumin and a single-chain antibody fragment (scFv).



22 **Figure 4:** Schematic illustration of a refolding device including a static mixer and plug flow reactor [39].

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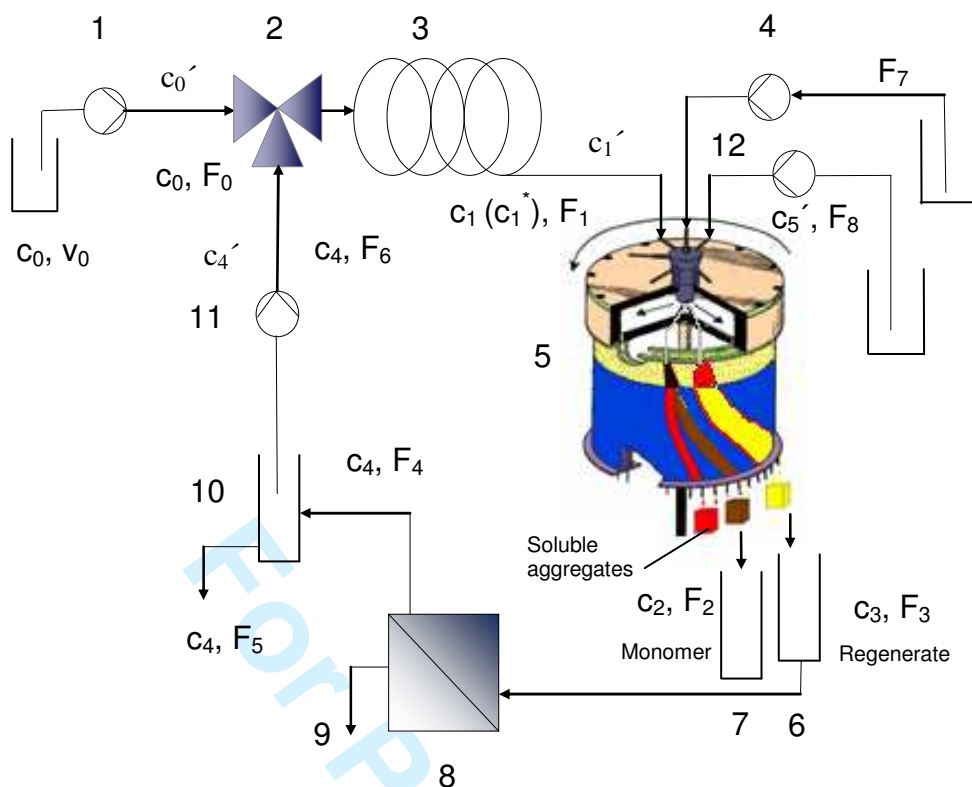


Figure 5: Experimental setup of continuous refolding by annular chromatography with recycling of aggregates. 1 feed pump delivering denatured protein; 2 mixer for blending of fresh feed with recycled feed after concentration by tangential flow filtration; 3 reaction loop; 4 eluent pump for the annular chromatography system; 5 annular chromatography system (a) 6 collecting device for regenerate fractions; 7 collecting device for monomeric protein fraction; 8 tangential flow filtration device; 9 permeate outlet; 10 vessel for collection of concentrated aggregates and 11 recycling pump; 12 pump for delivering regeneration buffer.

Table 1: Overview of additives for refolding of proteins, patents are bold

Additive	Concentration Range	Reference
L-Arginine	0.4 – 0.8 M	52,53,54
Urea	≤ 2.0 M	51
Guanidine hydrochloride	≤ 1.0 M	51
L-Argininamide	≤ 2.0 M	59,60
Ethylammonium nitrate (EAN)	0.5 – 50.0 %	61,62
Secondary and tertiary amines	≤ 1.5 M	63
Fluorine derivatives	0.5 - 95 %	64
Cyclodextrin (α -, β -, γ -)	5.0 - 10.0 %	66,67
Ionic liquid	0.25 – 5.0 M	75
Cycloamylose ^{*)}	16 mM	69 70
Alginate ^{*)}	0.25-3.0 %	71, 72
Reversed Micelles (AOT) ^{*)}		73

^{*)} stripping agent when a surfactant is used for stabilisation of protein

Table 2: Overview of patents and literature for various refolding techniques.

Homogenous and heterologous techniques	Patents	Literature reference
Solubilization of inclusion bodies	44,45,49,50	47,48
Batch dilution		32,33
Continuous dilution	28,29,34	30,31,32,33,41
Additives	59, 61,63,64,65,66,70	51,52,53,60,62,67,68,69,71,72,73,74
Oscillator and other mixing devices	36,38,39	37
On column refolding	92,93,105	91,94,96, 99,103
Immobilized folding catalysts	108*	104,109,110
High pressure refolding	82,84,86,87	52,81,85

*Application has been withdrawn

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