Spatiotemporal Control of Protein Refolding Through Flash-change Reaction Conditions

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Supporting Information Placeholder

ABSTRACT: Protein refolding is vital for protein production; however, a scalable method has not yet been developed. We introduce an innovative refolding approach using a flow microreactor (FMR) that allows precise control of buffer pH and solvent content. Using interleukin-6 as a model, the system yielded an impressive 96% pure refolded protein and allowed gram-scale production. This FMR system allows flash changes in reaction conditions, effectively circumventing protein aggregation during refolding. To the best of our knowledge, this is the first study to use an FMR for protein refolding, which offers a more efficient and scalable method for protein production . The study results highlight the utility of the FMR as a high-throughput screening tool for streamlined scale-up and emphasize the importance of understanding and controlling intermediates in the refolding process. The novelty of this approach is derived from the unique ability of the FMR to control both spatial and temporal aspects of protein refolding.

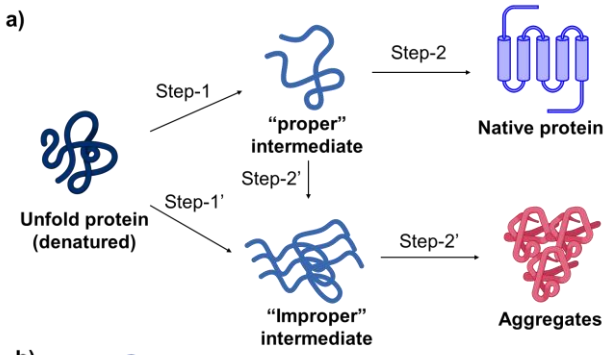
Protein function depends on primary, secondary, and tertiary structures.[1] The tertiary structure, influenced by external factors such as pH, is crucial. In recombinant protein production, proper refolding is necessary to restore this structure.[2] The major drawback of current refolding technologies is the formation of protein aggregates.[3-5] Optimization of folding conditions is a critical aspect of recombinant protein production and is typically achieved through laboratory experimentation and observation.[6-9] High-throughput screening methods are essential for streamlining the protein folding process. However, these methods are primarily used in early-stage research and have not been widely adopted by contract development and manufacturing organizations, making the development of a high-throughput and scalable protein refolding screening system a challenging task.

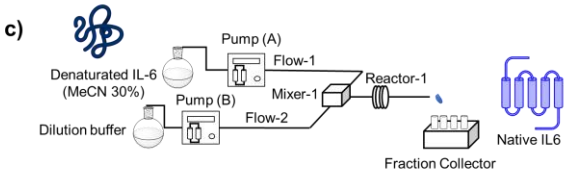
The continuous-mode flow reaction, which allows chemical reactions to take place in dedicated systems consisting of tubes, mixers, and pipes,[10-12] holds promise for achieving high-throughput protein refolding. However, its application in the biotherapeutic field is limited. In 2016, Rathore et al. demonstrated the viability of a continuous refolding system employing a coiled flow inverter[13], which boosted protein yield, although the purity level paralleled that achieved by batch-mode refolding.

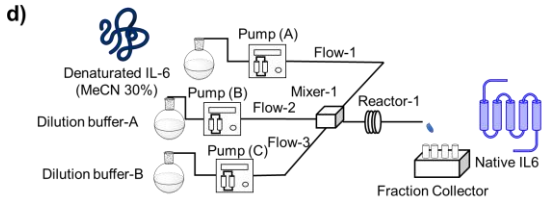
To overcome the limitations of current technologies, we used a flow microreactor (FMR) system for protein refolding. We studied the conformational changes of the cytokine interleukin (IL-)6 as a model,[14] at different pH and in various organic solvents[15, 16] to determine the most effective way to generate the optimal IL-6 conformation. IL-6 has a α-helix-rich structure and tends to dimerize and aggregate, making it a suitable model .

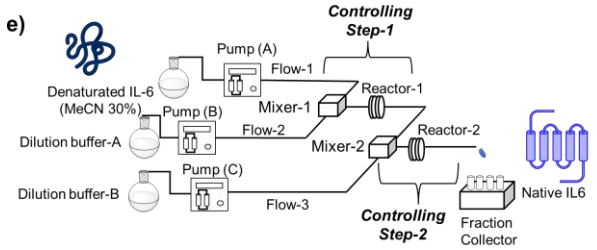
In general, protein refolding involves intermediates that ultimately lead to the native-state protein structure.[17] We hypothesized that during IL-6 refolding, “proper” and “improper” intermediates are formed, with only the former being converted into the monomer and the latter forming aggregates (Figure 1a, steps 1 and 2). For denatured IL-6, specific conditions are required to yield the proper intermediates; otherwise, improper intermediates are formed and aggregate. The proper form was found to be unstable and could be converted into the improper form if not properly managed. To address these challenges, an FMR system (Figure 1c–e) was introduced with the aim of capturing the proper intermediate and facilitating its conversion into a monomer. Different flow reactors were compared to determine the most suitable mixing system (Figure 1b–e). The standard batch dilution refolding method (Figure 1b), in which denatured IL-6 is mixed with a buffer, was used as a reference and was compared with a two-channel system (Figure 1c), a three-channel system (Figure 1d) allowing for varied mixing,

and a cascaded system (Figure 1e) designed for sequential mixing. All flow reactors achieved higher monomer yields than the batch method, with the three-channel setup yielding the best results (Figure 1f).









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Flow reactor system	Monomer by SEC analysis (%)
batch-mode	51
dual-channel microreactor	70
three-channel microreactor	60
cascaded-channel microreactor	74

Figure 1. FMR-mode refolding. (a) "Proper" vs. "improper" intermediates. (b) Batch-mode system. (c) Dual-channel microreactor. (d) Triple-channel microreactor. (e) Cascaded-channel microreactor. (f) Feasibility study summary.

Next, we attempted to identify the critical factors for using FMR as a high-throughput system. The cascade-lined FMR system consisted of two T-shaped mixers and two reactors, as previously reported (Figure 1e).[18] The reactor details are: lengths, reactor 1, 0.25 m, reactor 2, 0.25 m; diameter, 1×10^{-3} m; volumes, reactor 1, 0.196 mL, reactor 2, 0.196 mL; flow rate, 0.2 mL/min (IL-6), 1.8 mL/min (buffer A), and 2.0 mL/min (buffer B); total residence time, 8.83 s = 0.196 (mL)/2.0 (mL/min) + 0.196 (mL)/4.0 (mL/min). Refolding was completed in 8.83 s (residence time in reactors 1 and 2). This rapid reaction mode theoretically enabled screening more than 100 reaction conditions within 20 min.

To identify critical factors for refolding denatured IL-6, we conducted a feasibility study using an FMR system, exploring various parameters and conditions (Tables 1 and S1). Since various factors contribute to protein aggregation, we focused our investigation on pH and organic solvent content. Structural analysis of IL-6 revealed significant disorder (Figure S1), primarily due to its tendency to dimerize.[19] However, neutral pH stabilized refolded IL-6. Surprisingly, aggregation increased when denatured IL-6 was neutralized in step 1, indicating improper intermediate formation (entries 1–3). These results suggest that neutral pH can stabilize refolded IL-6, but does not necessarily induce proper intermediate formation. To determine the IL-6 aggregation ratio, we employed size exclusion chromatography (SEC) using a Superdex® 75 column composed of highly cross-linked agarose, using published analytical conditions.[20] To minimize potential adsorption to the column surface and ensure reproducible measurements, we added arginine to the mobile phase.[21] This allowed us to achieve improved separation with high recovery and to obtain accurate and reliable measurements of IL-6 aggregation. We explored various analytical conditions to identify the combination that provided the best resolution.

Analysis of entries 4 and 5 revealed that final buffer pH increased formation of monomers. Previous studies[21] on IL-6 has a narrow ionization window, making it particularly sensitive to pH conditions. Based on this understanding, we conclude that a pH of approximately 4.5 avoids protonation of the imidazole group of histidine residues and deprotonation of carboxylic residues,[22] stabilizes the proper intermediate of IL-6, and facilitates selective monomer formation.

To further improve monomer selectivity, we tested ways to reduce the organic solvent content in step 1. We found that dilution to pH 2.0 in step 1, followed by pH adjustment to 4.3 in step 2, was optimal, resulting in >81% monomer selectivity (entry 8). The type of dilution buffer employed and the counter ions used in step 1 had no noteworthy effects (entries 10, 11). We inferred that the monomer yield could be enhanced by reducing the acetonitrile (ACN) concentration while maintaining a pH of 2.0–3.0

during step 1 and elevating the pH to around 4.0–4.5 during step 2 (Figure S2).

Table 1. Feasibility study of FMR-mode refolding

Entry	Step-1				Step-2			Results
	Initial pH	Dilution buffer-A	Mixer-1 pH	Mixer-1 ACN conc. (%)	Dilution buffer-B	Mixer-2 pH	Mixer-2 ACN conc. (%)	Monomer (%)
1	2.5	50 mM Citrate-ACN (pH 3.0)	2.9	30	500 mM Acetate (pH 4.5)	4.3	1.5	53
2	2.5	50 mM Citrate-ACN (pH 4.5)	4.3	30	500 mM Acetate (pH 4.5)	4.5	1.5	42
3	2.5	50 mM Citrate-ACN (pH 6.0)	5.8	30	500 mM Acetate (pH 4.5)	4.6	1.5	35
4	2.5	50 mM Citrate-ACN (pH 3.0)	2.9	30	500 mM Citrate (pH 3.0)	3.0	1.5	42
5	2.5	50 mM Citrate-ACN (pH 3.0)	2.9	30	500 mM Acetate (pH 5.5)	5.1	1.5	38
6	2.5	0.1 % TFA in ACN-water (pH 2.0)	2.3	30	500 mM Acetate (pH 4.5)	4.3	1.5	54
7	2.5	0.1% TFA (pH 2.0)	2.2	1.5	500 mM Acetate (pH 3.0)	2.8	< 0.1%	69
8	2.5	0.1% TFA (pH 2.0)	2.2	1.5	500 mM Acetate (pH 4.5)	4.3	< 0.1%	81
9	2.5	0.1% TFA (pH 2.0)	2.2	1.5	500 mM Acetate (pH 5.5)	5.1	< 0.1%	60
10	2.5	50 mM Glycine-HCl (pH 2.2)	2.3	1.5	500 mM Acetate (pH 4.5)	4.3	< 0.1%	75
11	2.5	50 mM Citrate (pH 2.2)	2.3	1.5	500 mM Acetate (pH 4.5)	4.3	< 0.1%	75

Our initial assays showed that the refolding of suitable intermediates for IL-6 is delicate, with even subtle changes in conditions significantly altering aggregation. These findings led us to hypothesize that the lifetime of appropriate intermediates is extremely short and emphasized the need for rapid pH and organic solvent changes, using flash-change buffers) to obtain high-purity IL-6 monomers.

We determined that the FMR system is the most viable solution to address these challenges as it allows for easy adjustment of flow rates for flash changes and of the residence time in the FMR unit. We conducted additional screening studies to identify the optimal flow rate and residence time and generated a more comprehensive dataset (Table 2) to inform future process optimization efforts.

The residence time in step 1 was the most critical factor for forming IL-6 monomer, indicating that the appropriate intermediate is unstable and readily converts to the improper intermediate (entries 1–3). The flow rate to realize flash changes in the buffer was the most significant factor for improving monomer selectivity. Notably, the maximum flow rate required to maintain mixability, as assessed by the Villermaux–Dushman reaction,[23] resulted in >95% monomer selectivity (entry 8), representing a significant improvement. The flash buffer changes achieved by the cascade mixing system allowed for precise control of steps 1 and 2, which had suggested the existence of proper but unstable intermediates.[24]

Table 2. Exploration of flash-change buffer conditions

Entry	Step-1				Step-2			Results
	Flow rate of IL-6	Flow rate of dilution buffer-A	Reactor-1 length	Residence time	Flow rate of dilution buffer-B	Reactor-2 length	Residence time	Monomer (%)
1	0.2 mL/min	1.8 mL/min	5 cm	1.2 S	2.0 mL/min	25 cm	2.94 S	54
2	0.2 mL/min	1.8 mL/min	25 cm	5.9 S	2.0 mL/min	25 cm	2.94 S	81
3	0.2 mL/min	1.8 mL/min	50 cm	11.8 S	2.0 mL/min	25 cm	2.94 S	55
4	0.4 mL/min	3.6 mL/min	100 cm	11.8 S	4.0 mL/min	25 cm	2.94 S	80
5	1.0 mL/min	9.0 mL/min	100 cm	4.7 S	10.0 mL/min	25 cm	1.2 S	66
6	1.0 mL/min	9.0 mL/min	50 cm	2.4 S	10.0 mL/min	25 cm	0.59 S	84
7	1.0 mL/min	9.0 mL/min	25 cm	1.2 S	10.0 mL/min	25 cm	0.59 S	86
8	1.0 mL/min	9.0 mL/min	5 cm	0.2 S	10.0 mL/min	25 cm	0.59 S	96

Next, the system's scalability was assessed. Triplicate tests conducted at three scales (10 mg, 100 mg, and 500 mg) consistently produced a stable monomer yield of >95% and a deviation <1% (Table S3). A gram-scale preparation (starting from 1.3 g denatured IL-6) was explored with the aim of producing highly monomeric IL6 matching or surpassing the purity of commercial standards (>95%). The optimal residence time for this procedure was 0.79 s, allowing for the processing of the entire 1.3 g denatured IL-6 in approximately 4 h if the system was operated continuously. Post-refolding, tangential flow filtration and SEC purification were employed to eliminate contaminants, yielding >1.25 g IL-6 with >99% monomer purity, which is in line with commercial grade requirements (Table 3 and Figure 2). Moreover, minimal endotoxin presence was detected in the purified IL-6, suggesting that the closed reactor configuration consisting of tubes and pump may be effective in reducing endotoxin contamination risks and underlining the viability of the refolding system for potential application in pharmaceutical protein production.

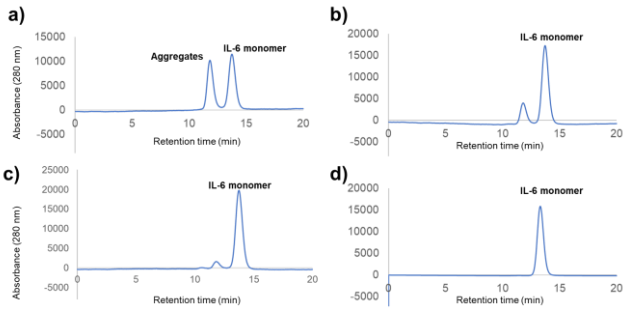

Figure 2. SEC-high-performance liquid chromatography analysis of refolded IL-6. (a) Batch-mode production (51% monomer). (b) Cascade-mode, without flash change (Table 1, entry 8: 81% monomer). (c) Gram-scale, cascade-mode with flash change (Table 2, entry 8: 96% monomer). (d) Gram-scale post-SEC purification (Table 2, entry 8: 99% monomer).

Table 3. Summary of gram-scale IL-6 production

Quantity	Residence time (Reactor-1 and -2)	Monomer % After Flow reactor	Monomer % Final product	Endotoxin conc.
1.25 g	0.79 S	96%	99%	<0.100 EU/mL

This study of the efficacy of the FMR system in refolding IL-6 highlighted the critical factors for refolding and revealed the existence of a proper but unstable intermediate. Effective management of this intermediate necessitates a flash-change tandem mixing system. A scaled-down manufacturing model combining the FMR refolding system with SEC purification yielded 1 g IL-6 with 95% monomeric purity. The success of the FMR system in refolding IL-6 underscores its potential applicability to diverse proteins. The system's high-throughput screening capacity can expedite the scale-up phase of protein production, conserving both time and resources. As it allows concurrent spatial and temporal control during refolding, the FMR system stands poised to revolutionize protein manufacturing, advancing protein therapeutic innovations and deepening our understanding of protein folding dynamics. The most important study finding may be the identification of intermediates pivotal in the transformation of proteins and the newfound capability to regulate them.

ASSOCIATED CONTENT

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24. To establish these monomer-selective conditions, our research group evaluated over 100 reaction conditions, including mixing system, pH, organic solvent ratio, ionic strength, and additives, as outlined in Table S1-S3 in the Supplementary Information.