Overview of refolding methods on misfolded recombinant proteins from *Escherichia coli* inclusion bodies

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**ABSTRACT**

Among bacterial expression system, *Escherichia coli* was the popular and widely used expression host due to its high rate expression trait. However, overexpression of recombinant protein in *E. coli* often found as inclusion bodies. While formation of inclusion bodies is beneficial in protein isolation from other cellular components, most of recombinant proteins from inclusion bodies are misfolded which have lost their biological activity. Protein refolding allows misfolded protein to rearrange into their native conformation which exhibit its biological activity, thus protein refolding become a pivotal step to recover active protein. However, protein refolding was affected by various factors; hence, screening of refolding condition was required to meet the optimal result. As a consequence, rapid and efficient characterization method is required to monitor the refolding performance. In this review, we will briefly give an overview about protein refolding method, chemical additives in protein refolding, and also provide insight in structural characterization to evaluate refolding performance.

### 1. INTRODUCTION

The expression of recombinant protein is an expanding field in biochemistry, providing various in-depth studies on protein expression and exhibits practical application in diagnostic, therapeutic, or other industrial use [1,2]. Among other expression hosts, *Escherichia coli* remain the preferable choice due to its ability to reach high-cell density culture and providing high-protein yield. However, expression in *E. coli* is lacking in post-translational modification, which become a disadvantage to protein that has pivotal recognition based on post-translational modification accessories such as glycosylation [3]. Furthermore, protein expression in *E. coli* tends to produce as inclusion bodies. Formation of inclusion bodies offers advantages in higher quantity of protein target and facilitates convenient isolation, yet it sacrifices the biological activity. Protein in inclusion bodies often found in misfolded form, therefore, refolding was required to rejuvenate its activity [2,4-7].

Protein refolding from inclusion bodies oftenly encompasses addition denaturant and removal of detergent. Denaturants such as guanidinium chloride or urea were added in solubilization of inclusion bodies, which helps misfolded protein revert back to its linear form. Later, removal of detergent helps to induce protein refolding [8]. The most common conventional methods in protein refolding include dilution and refolding, yet these methods are inexpensive and simple to be applied. Other methods involve chromatographic system, enzyme-assisted refolding, immobilization folding catalyst and artificial chaperones, and microfluidic chips were known to help in protein refolding [8-10]. However, the major drawback in protein refolding was formation of protein aggregates. Several strategies have been studied to reduce protein aggregates using chemical additives. Amino acids such as proline and arginine (Arg) show ability to inhibit aggregate formation, while polyol such as glycerol or glucose oftenly combined with aggregation inhibitor to stabilize proteins [11]. Challenge in protein refolding escalates while working with cysteine-rich proteins. The ability to form covalent bond from cysteine residue increases misfolding chance due to incorrect disulfide bridge. Therefore, addition of oxo-shuffling agents such as glutathione system to the buffer helps the refolding performance through reformation of disulfide bridge [12].

Since several factors are affected protein refolding results, screening of refolding condition often performed to meet the optimal refolding result. Since several factors are affected protein refolding results, screening of refolding condition often performed to meet the optimal refolding result using experimental design, such as response surface method which assists in refolding screening. Furthermore, using differential scanning fluorimetry (DSF)-guided protein refolding helps to evaluate refolding for large number of samples and rapid measurements. Moreover, *in silico* assistance using genetic algorithm (GA) guides in buffer and refolding condition determination for screening purposes [13]. As a result of refolding step, inactive protein was converted into its active form. Therefore, characterization on protein structure is required to evaluate the refolding performance. Evaluation on protein three-
dimensional structure using X-ray crystallography has been known in protein chemistry, yet the application for protein refolding evaluation is not favored due to time-consuming process. Therefore, another method such as Fourier transform infrared (FTIR)-ATR, circular dichroism (CD) spectroscopy, reverse-phase high-performance liquid chromatography (RP-HPLC), Raman spectroscopy, and size exclusion high-performance liquid chromatography (SE-HPLC) is more favored in refolding performance evaluation [14,15].

In this review, we will briefly discuss the conventional and latest protein refolding methods together with its chemical additives to better comprehend on protein refolding. In addition, we compile reports regarding refolding screening methods. We also summarize several techniques to evaluate refolding performance.

2. PROTEIN REFOLDING METHODS

Among refolding methods, simple dilution and dialysis are the conventional refolding methods, yet the practice is relatively simple and cost effective thus still widely used [8]. More advanced method using chromatography technique such as size exclusion chromatography (SEC), HIC, and affinity chromatography also can be applied to various proteins. However, aggregates formation and decreasing protein yield become challenges in refolding step. Therefore, determination of refolding methods and its optimization against specific protein need to be conducted experimentally [9,10].

2.1. Dilution Method

The dilution method is widely used to reduce the concentration of denaturing in the refolding stage because the method is simple. The dissolved protein is diluted 100–1000 times with a non-denatured refolding buffer. In this method, both the concentration of protein and denaturation will decrease. The protein concentration was kept at a low concentration of 1–100 g/mL to avoid protein aggregation. Denaturant has a higher diffusion coefficient than dissolved protein, so denaturant will diffuse faster than protein when dilution occurs. Various parameters that affect the refolding results are agitation rate, sample addition rate, addition time, and dissolved protein concentration. On a larger scale, such as an industrial scale, this method has several drawbacks, namely, it requires a large volume of container and refolding buffer also, requires additional steps to concentrate the protein. The dilution method can be carried out with several approaches including reverse dilution, flash dilution, and pulse dilution. In reverse dilution, the refolding buffer is added to the dissolved protein where generally, the dilution is done by adding the dissolved protein to the refolding buffer. In flash dilution, dissolved protein is added to the refolding buffer with one addition. Whereas in pulse dilution, a number of dissolved proteins are added gradually into the refolding buffer at successive time intervals [8,16-19].

2.2. Dialysis Method

Another simple method to reduce the concentration of denaturing agents is through dialysis. The protein concentration was almost constant before and after refolding with dialysis. Dialysis can be carried out in one or several stages, where single-stage dialysis is a simple method. The protein concentration is almost constant during the refolding process. The protein will begin to undergo refolding when the denaturing concentration decreases. Denaturant concentration will decrease with increasing dialysis time. However, folding and aggregation errors occur because of the contact between the exposed hydrophobic surfaces. This indicates that a rapid decrease in denaturing concentration will initiate aggregate formation as observed in the dilution method [8,20]. To overcome this problem, a stepwise dialysis method can be used. In stepwise dialysis, the denaturing concentration was gradually reduced from high, medium, to low concentrations. The dissolved protein will be equilibrated before the denaturing concentration is reduced again. A gradual decrease in the denaturing concentration of dissolved protein can result in high refolding efficiency. When denaturation in moderate concentrations of protein, often occurs the formation of aggregates and proteins that experience folding errors. Thus, reducing the dialysis time may reduce the formation of protein aggregations. In addition to aggregation, another challenge faced when using the dialysis method is the occurrence of non-specific absorption of proteins on the membrane [8,16,21].

2.3. Chromatography Methods

Refolding through chromatography columns was developed to increase refolding productivity without compromising yields, as well as for process scale-up in industrial fields. Protein immobilization in the chromatographic matrix helps isolate proteins spatially and reduces intermolecular interactions and promotes refolding. Refolding using chromatography has the advantage that it can be carried out at high protein concentrations and can reduce the purification steps because refolding and purification take place simultaneously [7,18,22,23].

Protein refolding through SEC occurs by exchanging the buffer to lower the denaturant concentration, while the proteins in the porous solid are developing into its native folding. Each protein constituent, aggregates, refolding intermediates, and native proteins are separated based on differences in the diffusion characteristics of the stationary phase. A gradual decrease in the concentration of denaturation and differences in the diffusion characteristics of each protein conformation will suppress the occurrence of aggregation. Thus, to improve this method is done by decreasing the urea gradient along with increasing the pH gradient. Other factors that affect the refolding result are dissolved protein concentration, sample volume, and flow rate. With the same dilution factor, SEC yields higher refolding yields than the dilution method as long as the dilution factor is below 40. Another advantage of this method is that it can create a loop system, where the intermediates and aggregates are separated from the native protein and added back to the feed solution so that the cycle is repeated. Hence, using this approach can greatly improve refolding results [8,18,20,21,24].

Similar principle also applied in other chromatography system, such as ion exchange chromato graphy (IEC) and affinity chromatography. These methods offer stronger protein-matrix interaction than SEC. In IEC, the refolding rate is slower than dilution method, but it offers higher native-like protein yield. However, since the retention is based on protein pH, non-specific interaction may occur and some impurities may also attach to the matrix which limit the amount of the protein loaded into the matrix. Affinity chromatography may overcome this challenge due to its specific interaction with protein. Common affinity chromatography used is immobilized metal affinity chromatography which has high-affinity divalent ion (Ni²⁺, Cu²⁺, or Co²⁺) immobilized on its matrix. Proteins with poly-His tag are easily bound to the column, this also offer purification step while conducting refolding. However, non-specific interaction through ionic interaction may hinder the process. Another affinity method is the use of chitin-binding domain as fusion partner, which has high affinity with cellulose matrix [8,18,22,24].

2.4. Immobilization Folding Catalyst and Artificial Chaperones

Refolding with a catalytic column is similar to how enzymes work in vivo. This approach is used for proteins that have disulfide bridges.
An oxidative chromatography column was developed which has three components immobilized on agarose: (1) GroEL minichaperone, which can prevent aggregation; (2) DsbA, which catalyzes the oxidation and exchange of disulfide bridges; and (3) peptidyl-propyl isomerase. This matrix was successfully used for refolding Cn5 scorpion toxin. In addition, a foldase oxidoreductase is immobilized in the separable matrix for scFv refolding and preventing protein aggregation. However, the application of this approach on an industrial scale is still limited due to its high cost [18,22,24].

Zeolite, which is a porous aluminosilicate crystalline compound, was reported as a new matrix for refolding. In industry, zeolites are widely used as cation exchangers and catalysts. Zeolites can capture dissolved protein strongly at high denaturing concentrations without the need for any markers. After the denaturation is removed, the protein is removed from the zeolite by adding a buffer containing a detergent such as polyethylene glycol (PEG) or Tween 20. As a result, protein aggregation can be efficiently suppressed before adsorption and then high refolding yields are obtained for both proteins with and without disulfide bridges [18,20].

Another approach used for refolding is that the function of chaperones is replaced by synthetic compounds. To mimic the allosteric characteristics of chaperones, an artificial chaperone-assisted (ACA) refolding method was developed, which involves two dilution steps. In the first stage, the dissolved protein is diluted with a buffer containing detergent to prevent aggregation by forming a protein-detergent complex. In the second step, the protein-detergent complex solution is diluted with a buffer containing detergent strippers, such as cyclodextrin. The first stage mimics the capture of the unfolded protein by GroEL and the second stage promotes the release of proteins, such as the allosteric function of GroEL. The ACA refolding approach is effective for refolding a wide variety of proteins. Several detergent releasing agents can be used such as oligomers, sugar polymers, and cyclodextrin modified polymers. In ACA refolding, the detergent releasing agent effectively removes the detergent so that the dilution ratio in the second refolding step will be much lower than in the dilution method.

2.5. Enzyme-Assisted Refolding

Protein folding can be done with the help of the enzyme urease to reduce the concentration of urea used. Urease is an enzyme that catalyzes the hydrolysis of urea into NH₃ and CO₂. Urease will hydrolyze urea which is used for dissolving inclusion bodies, decreasing urea will gradually make the protein undergo refolding. This method allows a slow and homogeneous reduction of denaturation without the need for a large volume of refolding buffer. Urease is immobilized on the separable resin to facilitate the separation of the refolded product. The limitation of this method is that it cannot be applied to denaturing agents other than urea [10,20].

3. CHEMICAL ADDITIVES IN PROTEIN REFOLDING

In most refolding systems, a competitive reaction between refolding and aggregation occurs in solution. Therefore, the condition of the refolding buffer solution is very important to increase the refolding result. In the case of refolding using the dilution method, there is a residual concentration of urea or GdnHCl in the refolding buffer. This low denaturing concentration is known to be able to maintain folding intermediates in a soluble and flexible state so that the refolding efficiency increases. In this regard, it is necessary to add additives or cosolutes to reduce aggregation and folding errors. At present, various additives have been reported as native protein stabilizers, refolding enhancers, and aggregation inhibitors. The refolding enhancer aids the interaction between proteins, thereby increasing protein stability. On the other hand, aggregation suppressors reduce side chain interactions of refolding intermediates without interfering with the refolding process [11,20].

3.1. Amino Acids

Arg hydrochloride (ArgHCl) or Arg is known to increase protein solubility and prevent protein aggregation during protein refolding.

Table 1: Some compounds used for protein folding.

| S. No. | Compound name | Function | Sources |
|--------|---------------|----------|---------|
| 1.     | GdnHCl        | 1. Can increases the refolding efficiency by maintain the folding intermediates in a soluble and flexible state. | [11,20] |
| 2.     | ArgHCl or arginine Arg | 1. Increases protein solubility and prevents protein aggregation during protein refolding. 2. Compared to GdnHCl, the amino and carboxyl groups of Arg form weaker hydrogen bonds with dissolved proteins and water so that Arg can act as an aggregation inhibitor due to its moderate binding to proteins. | [8,11,20,25] |
| 3.     | Proline       | 1. Inhibits protein aggregation by binding to folding intermediates and trapping the folding intermediates in the proline-formed supramolecule 2. Binds and stabilizes the refolding intermediates and reduces the hydrophobic surface. | [8,11] |
| 4.     | Polyol        | 1. At low pH can stabilize proteins in an acidic state by enhancing hydrophobic interactions that overcome electrostatic repulsion between charged residues 2. Creates an ideal environment where the rate of refolding increases while the rate of aggregation decreases | [8,11,20] |
| 5.     | Glycerol      | 1. Optimal glycerol concentration can increase the refolding rate which reduces aggregation | [8,11] |
| 6.     | Sorbitol      | 1. Can prevent protein aggregation by changing the structure and properties of the water around the protein molecule | [11] |
| 7.     | Eritrol       | 1. Stabilize proteins depending on the number of hydroxy groups it has | [11] |
| 8.     | PEG           | 1. PEG binds to intermediates in the protein folding pathway or interacts with the hydrophobic side chains of soluble proteins, thereby stabilizing the protein conformation and increasing the rate of protein folding. 2. Improves the formation of protein structure so that it can inhibit aggregation | [8,11] |

GdnHCl: Guanidinium hydrochloride, ArgHCl: Arginine hydrochloride, Arg: Arginine, PEG: Polyethylene glycol
Arg can increase protein folding yields with effective concentrations ranging from 0.4 to 1 M. The molecular mechanism of Arg is still unclear although experimentally it has shown its effectiveness in preventing aggregation. ArgHCl has a guanidinium group (Gdm) on the side chain which resembles GdnHCl. Even though they both have Gdm’s, the denaturing effect of ArgHCl is more moderate than that of GdnHCl, so it is better used for the refolding process. The crystal structure of the lysozyme-Arg complex indicates that Arg interacts on a different side with GdnHCl and slightly interacts with hydrophobic amino acids on the protein surface. These interactions

| Methods                          | Information                                               | Amount of protein needed | Advantages                                                                 | Disadvantages                                                                                           | Sources |
|----------------------------------|-----------------------------------------------------------|--------------------------|--------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------|---------|
| CD                               | - Far UV-C: secondary structure composition               | Far UV-C: 0.25 g/L       | 1. Measurements can be performed on physiological buffers 2. Non-destructive 3. Measurement time 30 min or less | 1. No specific residual information 2. Incompatible with high denaturing concentrations 3. Cannot use non-detergent sulfobetaine (NDSB) | [14,15] |
|                                  | - Near UV-C: Tertiary structure composition               | Near UV-C: 2.5 g/L       |                                                                          |                                                                                                           |         |
| Dynamic light scattering         | - Tertiary and quaternary structure                       | 0.05 g/L                 | 1. Rapid 2. No calibration or buffer blanking required 3. Can be used for all types of protein                          | 1. Low resolution 2. Samples must be free of other particles                                           | [14,15] |
| Dissolved oxygen and redox probes| Mild sensor to observe protein refolding with disulfide bridges | Depends on protein concentration | 1. Fast 2. Simple 3. Relatively inexpensive 4. DO sensor can be related to product quality | 1. Technical issues such as signal-to-noise comparison and probe deposition                              | [15]    |
| ESI-IMS-MS                       | Observing oxidation processes and aggregation studies     | ~1.5 g/L for protein with MW 50 kDa | 1. Analyze and quantify of conformational mixtures of proteins during refolding (such as different disulfide bridges pairings) | 1. Expensive equipment 2. Not suitable for non-volatile samples                                          | [15]    |
| Extrinsic fluorescence           | Tertiary and quaternary structure                         | ~0.015 g/L for protein with MW 50 kDa | 1. Sensitive 2. Suitable for selection | 1. The dye that appears may interfere with protein aggregation                                            | [15]    |
| ATR-FTIR                         | secondary structure                                       | > 0.01 g/L               | 1. Tolerance to salt and sample turbidity 2. Can be used for all proteins 3. Highly precise wavelength | 1. Not suitable for real-time monitoring                                                               | [15]    |
| NMR                              | Tertiary and quaternary structure                         | > 25 g/L for protein with MW 50 kDa | 1. Possible for real-time applications 2. Non-destructive 3. Structures can be analyzed in native conditions | 1. Requires a large number of samples 2. Limited to proteins with small size (< 40 kDa) or protein fragments | [14,15] |
| RP-HPLC                          | Primary structure and unfolded protein                    | > 0.3 g/L                | 1. Rapid 2. High resolution | 1. High temperatures during analysis can cause aggregate formation                                         | [15,27,28] |
| SE-HPLC                          | Quantitative analysis                                    | 0.012 g/L                | 1. Non-destructive 2. Sensitive 3. Solvent conditions do not cause denaturation | 1. Limited dynamic range 2. Inaccurate due to changes in size distribution                               | [15]    |
| Raman spectroscopy               | secondary structure                                        | > 1 g/L > 0.08 g/L for protein quantification | 1. Sensitive and selective to structure | 2. Expensive equipment (CW-UV laser) and complicated instrumentation                                      | [15]    |
| Zeta potential analysis          | Primary structure and early stage of refolding            | 10 nm: > 0.5 g/L 10–100 nm: > 0.1 g/L 100 nm–1 μm: > 0.01 g/L | 1. Simple 2. Fast | 1. Very sensitive to dust 2. Unable to detect secondary structure changes during secondary structure formation | [15]    |

CD: Circular dichroism, ESI-IMS-MS: Electrospray ionization-ion mobility spectrometry-mass spectrometry, ATR-FTIR: Fourier transform infrared, NMR: Nuclear magnetic resonance spectroscopy, RP-HPLC: Reverse-phase high-performance liquid chromatography, SE-HPLC: Size exclusion high-performance liquid chromatography
include electrostatic, hydrophobic, and cation interactions. When compared to GdnHCl, the amino and carboxyl groups of Arg form weaker hydrogen bonds with dissolved proteins and water. Thus, Arg might act as an aggregation inhibitor due to its moderate binding to proteins [8,11,20,25].

The role of proline was first studied in in vitro refolding of bovine carbonic anhydrase. Proline inhibits the aggregation of bovine carbonic anhydrase and allows the protein to return to its original structure. It is proposed that the protein inhibits protein aggregation by binding to the folding intermediate and trapping the folding intermediate in the proline formed supramolecule. Proline is used during the refolding of egg white lysozyme, Arg kinase, and aminoacylase. Proline (1 M) also acts as an agent that helps creatine kinase (CK) folding. It further demonstrated that proline might bind to and stabilize the refolding intermediates and reduce the hydrophobic surface of CK, thereby inhibiting protein aggregation and increasing the final CK yield. The hydrophobic nature of proline masks the hydrophobic region of the protein, where this amino acid effectively suppresses protein aggregation [8,11].

3.2. Polyols
Polyol compounds have many types depending on the length of the carbon chain and the number of hydroxy groups. Polyols are used as additives to increase the folding yield and stabilize the protein structure. The addition of polyols makes the structure more compact, for example, in RNase A. Furthermore, polyols can stabilize proteins in an acidic state by enhancing hydrophobic interactions that overcome electrostatic repulsion between charged residues at low pH. The addition of these additives creates an ideal environment in which the rate of refolding increases while the rate of aggregation decreases. Although the addition of a stabilizer can improve the coating yield, protein aggregation still occurs simultaneously. Therefore, this type of additive is always used in combination with aggregation inhibitors such as Arg [8,11,20].

Glycerol is known as a protein stabilizer and its stabilization mechanism has been described by Timasheff. Glycerol leads to increased hydrophobic interactions as a result of increasing the amount of solvent around the protein. Glycerol has a moderate stabilizing effect compared to other polyols. Unlike other polyols, glycerol has the unusual property of lowering the surface pressure of water but increasing its viscosity. Glycerol concentration that is too high will reduce the refolding rate which causes aggregation so that the glycerol concentration used needs to be optimized [8,11].

Sorbitol is another polyol that can prevent protein aggregation. Sorbitol most likely exerts an influence on folding by changing the structure and properties of the water around the protein molecule. Erythritol is known to have lower stabilizing characteristics than sorbitol. The study of citrate synthase kinetics during refolding with the addition of erythritol and other polyols showed that the ability of polyols to stabilize proteins depends on the number of hydroxy groups possessed [11].

PEG is one of the most versatile (water soluble) polymers for refolding recombinant proteins and stabilizing proteins by chemical modification. Like other polyols, PEG can stabilize protein conformation and increase protein refolding rate. PEG improves the formation of protein structure so that it can inhibit aggregation. During the refolding process, PEG binds to intermediates in the protein folding pathway or interacts with the hydrophobic side chains of the soluble protein [8,11]. Table 1 summarizes several chemical additives that can be used in protein folding.

4. APPROACHMENT ON REFOLDING MONITORING TECHNIQUE
Protein refolding is a bottle neck in retrieving biologically active protein which has further application in diagnostic or clinical therapy. While protein refolding methods are varied, each method needs to be optimized to obtain high-yield native protein. To evaluate the refolding performance, structural characterization is required. However, obtaining 3D structure through crystallization is time consuming and inefficient for rapid screening. Therefore, approachment characterization was used to evaluate refolded protein such as FTIR-ATR, CD spectroscopy, RP-HPLC, Raman spectroscopy, and SE-HPLC [14,15]. Nevertheless, while working on large number of samples, those techniques become inefficient. Another approachment using DSF-guided protein refolding gives rapid evaluation against large number of sample for refolding screening [26]. Other approachment of selecting refolding method was reported using in silico assistance using GA. This in silico approachment helps to guide refolding methods and screening of buffer condition, which supports comprehensive experimental design [13]. Otherwise, using RSM experimental design to evaluate refolding factors (pH, buffer, additives, and temperature) also helps in refolding screening. Table 2 summarizes several methods that can be used to monitor protein folding.

5. CONCLUSION
Expression of recombinant protein in E. coli is favored due to its high-yield product. However, its nature to form inclusion bodies during the process becomes a challenge, since inclusion bodies are mostly biologically inactive. Solubilizing the insoluble inclusion bodies and further refolding processes are required to obtain an active protein. The method for protein refolding is vary, such as dilution, dialysis, chromatography-based method, usage of immobilization folding catalyst, and artificial chaperones, and also enzyme-assisted refolding is among the available methods which offer different and unique advantages and disadvantages. Chemical additives also play role to improve the refolding process. Amino acid and polyols oftenly added to prevent protein aggregation, thus improving the soluble protein yield. Amino acid and polyols oftenly added to prevent protein aggregation, thus improving the soluble protein yield, while oxo-shuffling agents such as GSH/GSSG, β-mercaptoethanol, and DTT sometimes used to promote disulfide shuffling to obtain proper disulfide bridge pairs. Monitoring the refolding process was conducted by characterizing the protein structure. However, performing a rapid and accurate characterization sometimes become a challenge, in fact, both criteria are occasionally exchanging each other. Therefore, in this review, we briefly discussed protein refolding along with its structural characterization method.

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All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual
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The authors report no financial or any other conflicts of interest in this work.

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This research did not contain any studies involving animal or human participants, nor did it take place on any private or protected areas.

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