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Chapter 5

Solid-Phase Cell-Free Protein Synthesis to Improve Protein Foldability

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1. Introduction

Proteins are the most abundant molecules in biology which control virtually every biological process on which our lives depend. Therefore, understanding how newly synthesized proteins folds into the correct native structure and achieve their biologically functional states inside the cell is of paramount importance. Most of what is currently known about the process of protein folding has been studied by analyzing proteins outside the cells in a 'dilute solution' under *in vitro* conditions. The pioneering work on the creation of cell-free (*in vitro*) protein synthesis (CFPS) reported by Nirenberg and Matthaei in 1961 has been a powerful and ever expanding tool for large-scale analysis of proteins [1]. In general, these systems are derived from the crude extract of cells engaged in a high rate of protein synthesis and are consist of all the macromolecular components required for translation of exogenous mRNA which are added separately in the system. The cell-free system offer several advantages over traditional cell-based (*in vivo*) systems which are specially not good at making exogenous proteins and those which are toxic to the host cell, undergoes rapid proteolytic degradation or forms inclusion bodies. Cell-free system provides the ability to easily manipulate the reaction components and conditions to favor protein synthesis, decreased sensitivity to product toxicity and suitability for miniaturization and high-throughput applications. With these advantages, there is continuous increasing interest in CFPS system among biotechnologists, molecular biologists and medical or pharmacologists. However, CFPS systems rely on the correct folding of the expressed polypeptide chain into a fully functional three-dimensional protein. Thus 'foldability' of expressed protein in a cell-free system is one of the most challenging conundrums of CFPS science.

The folding issue (misfolding or aggregation) is believed to be caused by excessive collision between growing peptide chain and with other macromolecular components of cell-free

system. It is estimated that the total concentration of macromolecules such as proteins, nucleic acids, ribosomes and carbohydrates in the crude cell extract is ranged from 300 to 400 mg/mL that occupy about 30% of total cytoplasmic volume [2]. For easy understanding, if 30% of the volume of a cube is filled with macromolecules of a given size, uniformly distributed, then there is virtually no volume available for additional molecules of a similar size. This leads to 'macromolecular crowding' effect which can result in surprisingly large qualitative and quantitative effects on both the thermodynamic and kinetic of interactions among macromolecules. For example, it can favor the association of macromolecules which may lead to a dramatic acceleration in the rate of protein aggregation (a huge variety of diseases share the pathological feature of aggregated misfolded protein deposits such as formation of amyloid fibrils has a central role in the pathogenesis of Alzheimer disease) [3]. Second, crowding also limits the diffusion of molecules that limits the conformational flexibility of growing polypeptide chains, adding complexity to folding and multimerization reactions. Although CFPS is routinely carried out in relatively dilute solutions but yet the commonly used CFPS systems are estimated with a relatively crowding environments containing ~5% (w/v) of macromolecules [4]. Very recently, the inhibition of cell-free translation of *Rluc* mRNA was confirmed under macromolecular crowding conditions created by adding various biocompatible crowding agents. Interestingly, these crowding agents were observed to show an opposite effects on cell-free transcription reactions [4]. This study confirms that a macromolecule crowding may lead to terminal misfolding and therefore determine the folding rates. Thus protein folding which is crucial to the function of proteins requires controlled handling of translation reaction in CFPS system. In this stream, consideration of the protein behavior in their intracellular milieu is crucial. This chapter presents a novel approach, called solid-phase CFPS, which provides mimetic conditions of an intracellular milieu to facilitate efficient cell-free protein translation of more functionally active proteins.

2. Co-translational protein folding: What we can learn?

Protein synthesis is the universal mechanism for translating the genetic information into functional information in all kingdom of life and all synthesized proteins have in common to fold and express their biological activity. The machine which carries the protein synthesis is the ribosome, a large RNA-protein complex. However, the fundamental understanding of how does the ribosome move along an mRNA and how the linear amino acid sequence of a growing polypeptide chain folds correctly into its unique three-dimensional structure is still not completed. It is widely believed that protein folding generally begins during translation on the ribosome, called 'co-translational folding' [5-7]. This implies that the N-terminal part of a growing polypeptide starts its folding as soon as it has been synthesized, prior to the completion of entire polypeptide chain by the ribosome (see Fig.1). The experimental testing of this elegant idea was already begun in the early 1960s and today there is substantial experimental support for the co-translational folding hypothesis. Very recently, an efficient co-translational folding has been demonstrated by using an engineered multidomain fusion protein [8]. In one another study, the folding yield of fluorescent protein was compared

between ribosome-released GFP and chemically denatured GFP. The yield of native fluorescent GFP was dramatically higher with co-translational folding [9]. Although encouraging, but yet many details of co-translational folding pathway remain unanswered. For example, since the fact that the polypeptide synthesis requires many seconds (50-300 residues/min) and the folding occurs in much less than one second (or microsecond-level), there must be formation of compact structures and/or intermediates in the process of protein synthesis. So, what types of structures are these and how they effects on the folding efficiency of newly synthesized protein is still remain elusive.

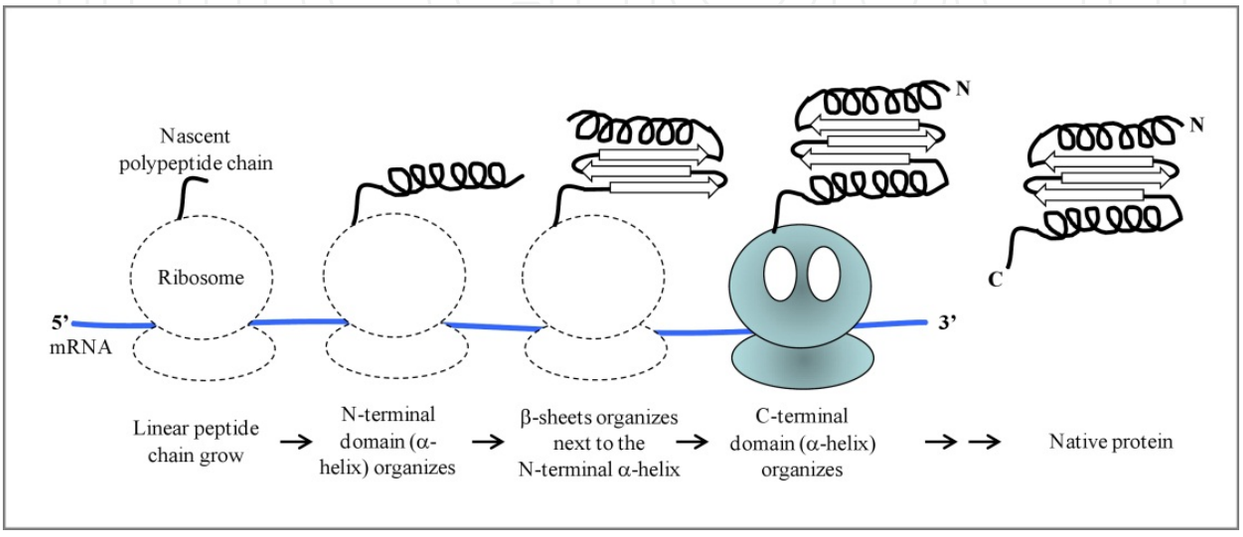

Figure 1. A cartoon representation of 'co-translational folding' of a growing polypeptide chain on the ribosome.

The ribosome serves as a platform for co-translational folding. A crucial process is the decision whether the folding occurs in the cytosol or across the membranes (eukaryotic endoplasmic reticulum (ER) membrane or bacterial plasma membrane). Eukaryotic co-translational protein translocation involves the interaction of signal recognition particle (SRP) with ribosomes. The SRP recognize the hydrophobic signal sequence at the N termini of nascent peptide chains as they emerge from the exit tunnel of ribosome and then SRP-RNC (ribosome–nascent chain) complex interacts with the ER membrane-bound SRP receptor to delivers nascent peptide chain to the ER membrane. This process slowing down chain elongation and lead to a transient arrest of translation. Once ribosome engages a proteinaceous channel located at the ER membrane, only then protein synthesis is resumed and nascent protein are co-translationally injected into the ER lumen. So, what we understand that slowing down the translation rate (as a result of co-translational process) may improve the folding efficiency of newly synthesized proteins. It has been observed that protein synthesis speed is faster in bacteria than in eukaryotes. In *E. coli*, polypeptide synthesis rates vary from 10 to 20 amino acids per second [10] but it is considerably slower (3 to 8 amino acids per second) in the eukaryotes [11]. Presumably, this might be the reason why the eukaryotic cytosol appears to be highly capable of folding proteins efficiently (as a result of co-translational folding) whereas folding of protein is delayed relative to their synthesis in the

bacterial cell. It is recently highlighted that a single codon mutation in mRNA that alters the translation rate can lead to a dramatic increase in the folding yield [12]. Thus, the speed of protein synthesis can affect protein folding pathways. And if this is true, then controlling the polypeptide synthesis rate would be promising step to improve the protein foldabiltiy in the CFPS systems. Since both the ribosomes and mRNA templates in the CFPS are not in a stationary mode (as they are in cell-based system represented by endoplasmic reticulum membrane-bound ribosome), providing a similar environment by introducing solid-phase chemistry would help to create co-translational protein folding in the CFPS systems.

3. Solid-phase versus solution-phase chemistry for protein synthesis

Solution dynamics (representing diversity of molecular conformations and motion) of biological macromolecules (e.g., DNAs, mRNAs) has been described by using nanosecond molecular dynamics or X-ray scattering approaches [13,14]. These studies suggest conformational variation including semi-stable or unstable structures having short life times is a general functional feature of these macromolecules and this is profoundly influenced by their environment, such as small changes in the concentration of solutes or salts can radically alter the properties of DNA/mRNA in the solution. These dynamics, such as spatial and temporal dynamic of mRNA movements that undergoes many conformational rearrangements and so an integral part of cell-based protein synthesis, however, may not require in the cell-free systems and thus should be avoided in the cell-free reactions. Secondly, exogenous mRNAs are extremely labile in nature and thus are apt to be degraded by contaminating nucleases that are inherently present in the crude cell extracts and thus the protein synthesis reaction is inhibited over time. Third, since CFPS carried out in relatively dilute concentrations, the ribosome turnover is likely less compared with the cell-based system. In order to exploit these issues, here we introduced solid-phase chemistry for the CFPS systems where the diffusional migration of key molecules (e.g., mRNAs or ribosomes) is restricted in a defined area to improve the positive reactions in a pseudo-first order fashion (see Fig.2).

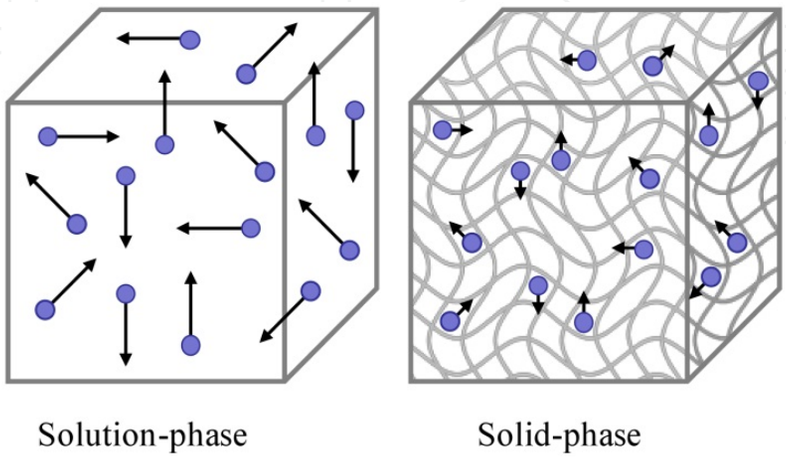

Figure 2. A schematic drawing of diffusional migration in solution-phase and solid-phase reactions. Blue circle represent the CFPS reactants.

Compared with solution-phase reaction, in which the reactants of CFPS are dispersed in a dilute solution, advantages of solid-phase CFPS reaction includes: (i) improved stability: the boundaries stabilized and protect biomolecules by capping the free terminal ends against nucleases degradation; (ii) higher local concentration: the local concentration of the reactants can be greatly increased in solid-phase, a condition that cannot be realized in the solution-phase because of the extra volume of the solvent and the fixed solubility of template DNAs/mRNAs. For example, ribosome-turnover can be increased to find its next substrate in solid-phase reaction; (iii) post-reaction steps: it become easier to perform purifications or remove excess reactant or byproducts from the reaction; (iv) co-translational folding: it mimic the cell-bases system by introducing a diffusion barrier which significantly reduces the reaction rate and improve the co-translational folding. A schematic drawing of solid-phase CFPS is outlined in Fig.3 and compared with solution-phase CFPS and cell-based system. Here, we should recall that protein synthesis is compartmentalized in the cell-based system and secretory/integral proteins being synthesized on endoplasmic reticulum (ER) by trafficking of the ribosome and mRNA from the cytoplasm to the ER membrane. Therefore, solid-phase CFPS where mRNAs are immobilized on a solid surface provides the similar environment with the cell-based system by controlling the reactions in a similar stationary mode using surface-bound mRNA, and this may help to direct protein folding.

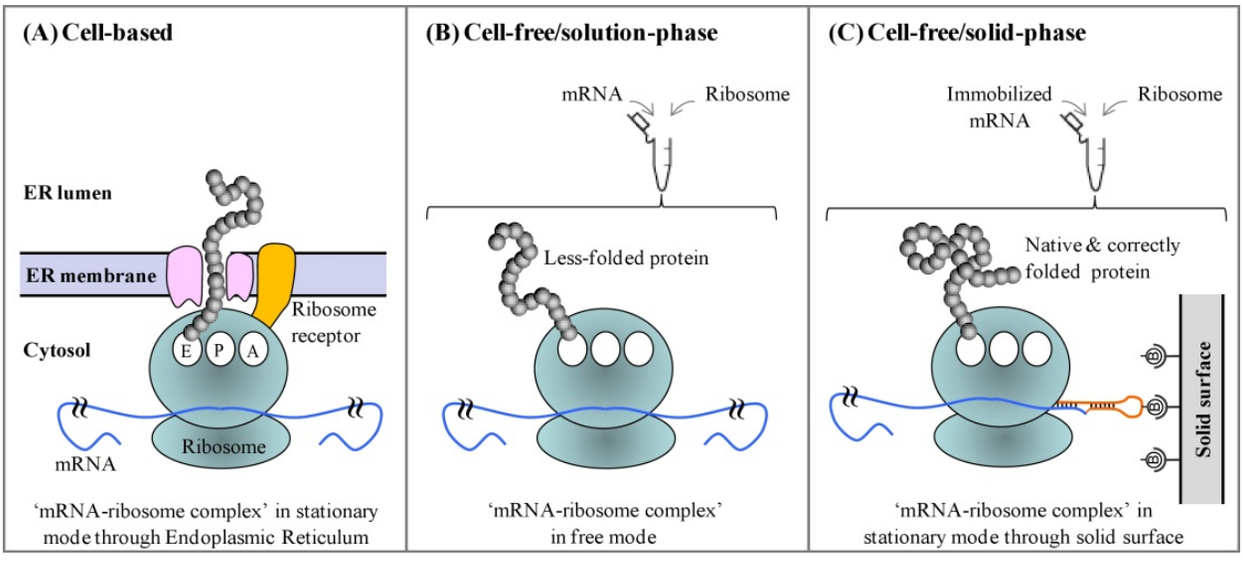

Figure 3. Schematic representation of protein synthesis *in vivo* (A) conventional in vitro solution-phase (B) and novel in vitro solid-phase (C).

The solid-phase approach was first invented by Bruce Merrifield in 1963 in an effort to overcome difficulties inherent to the liquid-phase synthesis of peptide [15]. Later, the immobilization of biomolecule and synthetic solid-phase approaches have been successfully

aided research for a widespread applications for both pre-translated biomolecules such as RNA/DNA and post-translated biomolecules such as protein/enzymes including SNP genotyping [16], DNA amplification [17], differential display [18], *in vitro* transcription [19], immunoassay [20,21], and others while promoted the development of microfabrication [22,23], high-throughput screening and automation strategies in many areas including proteomics. Very recently, a hydrogel-based system was introduced that improved the efficiency of CFPS up to 300 times than solution phase-based system [24].

The simplest method for immobilization of biomolecules is physical adsorption between the molecule of interest, e.g., protein, and solid surface [25]. However a more stable and reliable mean of immobilization is a bonding or linkage between the molecule of interest and molecules of the solid support [26]. To date, several methods have been reported to bind the functional biomolecules with ligands onto glass, agarose bead gels and magnetic particles. Among these, the covalent nature bonding affinity has advantageous over non-covalent bonding in the ability to orient the immobilized molecule in a defined and precise fashion for forthcoming reactions. The affinity of biotin for streptavidin is one of the strongest and most stable known in biochemistry [27]. Moreover, a wide range of immobilizing materials and binding modes allows a great deal of flexibility in order to design a specific bond with specific physical and chemical properties such as charge distribution, hydrophobic/hydrophilic, etc. In this chapter, we highlight our new approach of solid-phase protein synthesis to improve the stability and foldability of CFPS systems.

4. General concepts for solid-phase CFPS

In order to exploit the above issues, a novel solid-phase CFPS was described to produce proteins in their native folded-state which is schematically outlined in Fig. 4 [28]. The requires the template (mRNA) in a stationary phase, which is achieved by immobilizing the mRNA molecules to a solid-surface prior to translation. In order to perform solid-phase translation, the immobilization of mRNA must satisfy several requirements: (i) mRNAs should be attached efficiently to the solid surface via a 3′-UTR end linkage, (ii) the integrity of the mRNAs should not be affected by immobilization, (iii) the availability of the free 5′-end of the mRNA must be sufficient for translation and (iv) the properties of the solid-surface must be compatible with translation. These are achieved by coupling the mRNA of interest to a solid surface via ligation to a synthetic biotinylated DNA oligomer which is then immobilized to streptavidin-coated paramagnetic beads. An efficient ligation is an essential part of solid-phase translation and for this purpose we have engineered a synthetic linker-DNA molecule (see Fig. 4A). To perform an efficient ligation between the mRNA and linker-DNA molecules, the 3′-ends of the mRNAs are first hybridized to the linker-DNA and then incubated with T4 RNA ligase. This reaction is efficient even at low concentrations of substrates as it is based on quasi-intramolecular ligation. In the next step of solid-phase translation, the bead-bounded mRNA molecules are incubated in a cell-free translation system (Fig. 4B).

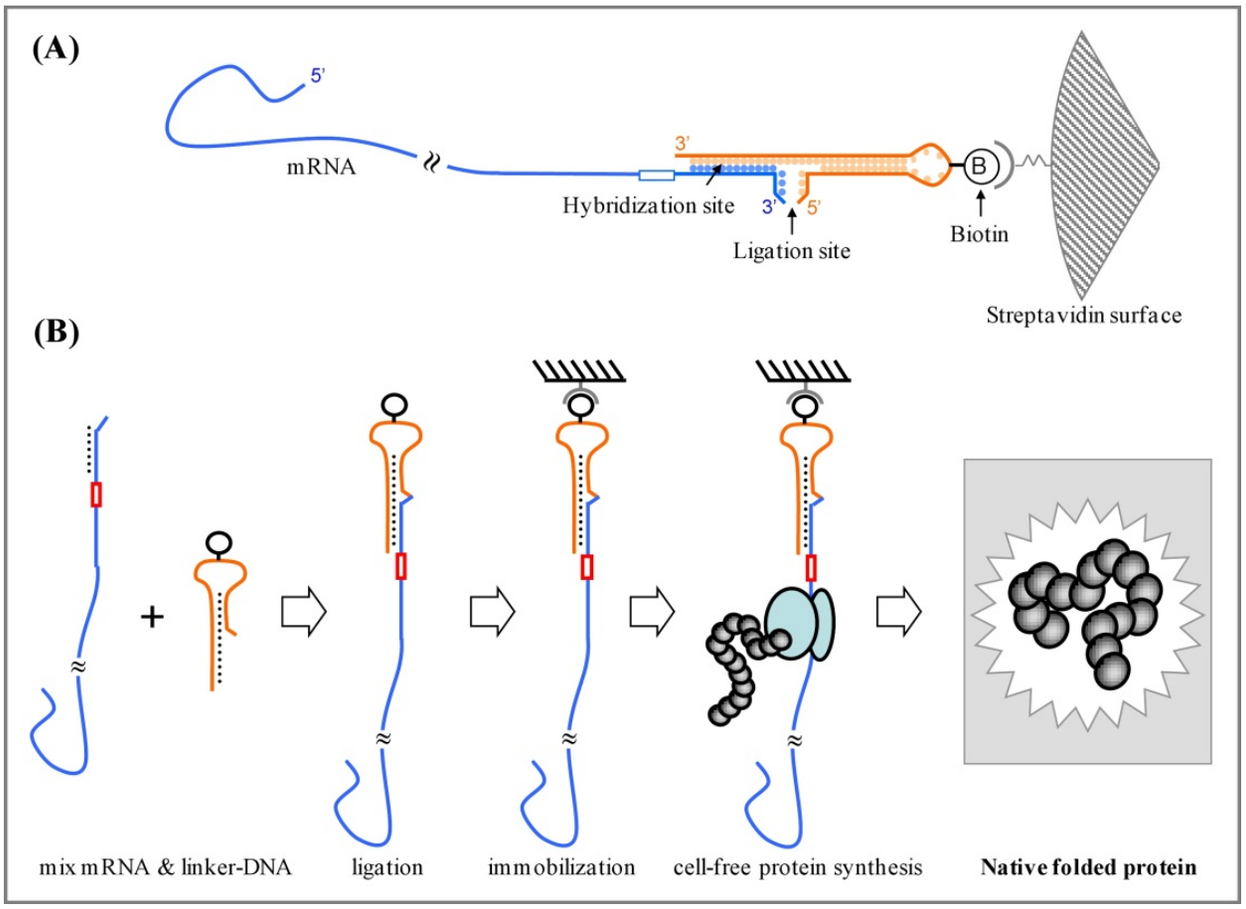

Figure 4. Schematic representation of a novel solid-phase cell-free protein synthesis for synthesizing native and correctly folded protein.

5. Yield of natively folded proteins by solid-phase CFPS

To demonstrate the performance of solid-phase translation system, FP (fluorescent proteins: GFP, green fluorescent protein and mCherry) was chosen as the model proteins. A T7 promoter driven DNA template encoding mCherry with a stop codon was constructed and amplified with biotinylated primer. The PCR products were then immobilized onto streptavidin-coated paramagnetic beads. Following cell-free couple transcription/translation reaction, the beads were separated and the supernatant was analyzed by native SDS-PAGE. To compare the performances of solid-phase and solution-phase systems, an identical quantity of free PCR products without immobilization was processed in parallel. The original fluorescence of the folded mCherry protein was successfully resolved by SDS-PAGE as a major band of ~28 kDa (see Fig. 5A). The RFU (relative fluorescence units) values representing the foldability of mCherry bands were monitored by a fluorescence imager. The average results obtained by three successive experiments clearly show that synthesis of mCherry using novel solid-phase system was at above 2-fold of the solution-phase system (Fig. 5B).

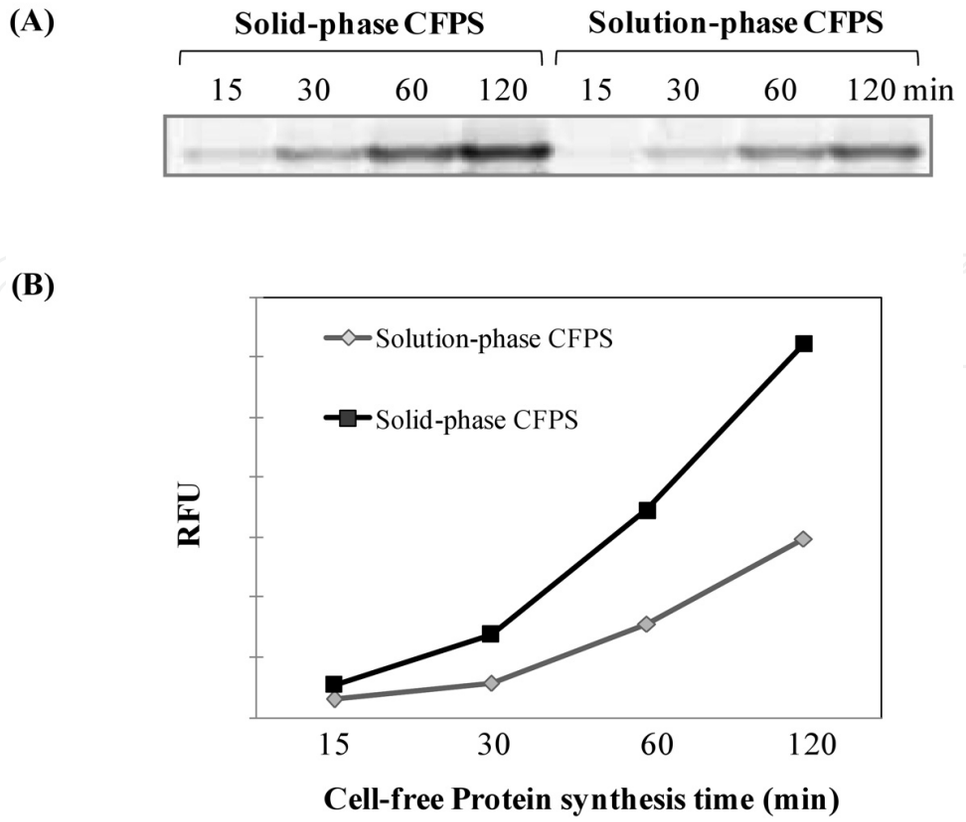

Figure 5. Comparison of the protein synthesis of correctly folded mCherry by solid-phase and solution-phase CFPS. A native SDS-PAGE analysis and quantitative measurements (B).

Indeed, it was surprising to see that by simply converting the free DNA template to surface-bounded template, the efficiency of protein synthesis using coupled transcription/ translation system was much improved. To understand this further, we studied the underlying mechanisms by investigating cell-free translation separately. For this purpose, a T7 promoter driven mRNA template encoding GFP with a stop codon and short stretch of complementary sequence of linker-DNA at the 3′-terminus was constructed (as shown partly in Fig.4A). This template was then ligated to linker-DNA and immobilized onto streptavidin-coated paramagnetic beads. Following cell-free translation in a wheat germ-based system, the beads were separated and the supernatant was analyzed quantitatively by SDS-PAGE and qualitatively (i.e., correct folding) by a fluorescence microplate reader. To compare the performances of solid-phase and solution-phase systems, an identical quantity of free mRNA-template without ligation or immobilization was processed in parallel. To quantitatively compare the production between the solid- and solution-phase methods, GFP was expressed using fluorescently labeled lysine residues. Translated products were heated at 70°C for 5 min for complete denaturation and removal of the original fluorescence of the folded GFP protein, and resolved by SDS-PAGE. Heat-denatured (non-fluorescent) GFP migrates as a major band of about 27 kDa (Fig.6A, right two lanes). The intensity of FluoroTect labeled GFP bands were monitored by a fluorescence imager. The average results obtained by four successive experiments clearly show that production of GFP using our solid-phase system was at about 15% of the levels of the liquid-phase system (Fig.6B

inset, white columns). However, the quality analysis, i.e., foldability, of the GFP, for these two systems obtained by measuring the intensity of original green fluorescence, (Fig.6B inset, gray columns) showed similar results. The RFU (relative fluorescence units) values representing the foldability of GFP were directly measured using a fluorescence microplate reader, and for the solid-phase system was about 80% of the liquid-phase system. This suggests that although the production of GFP using the solid-phase approach is considerably less compared with the liquid-phase method, the proteins produced in the solid phase are up to four-fold more biologically active after normalization (Fig.6B). To confirm this finding, the solid-phase products were removed from the beads and then analyzed together with solution-phase products by SDS-PAGE. The results showed a 37 kDa GFP product from the solid phase reaction, which is shifted upwards from the denatured position predicted for its theoretical mass (27 kDa) due to its native folding (Fig.6A, left two lanes).

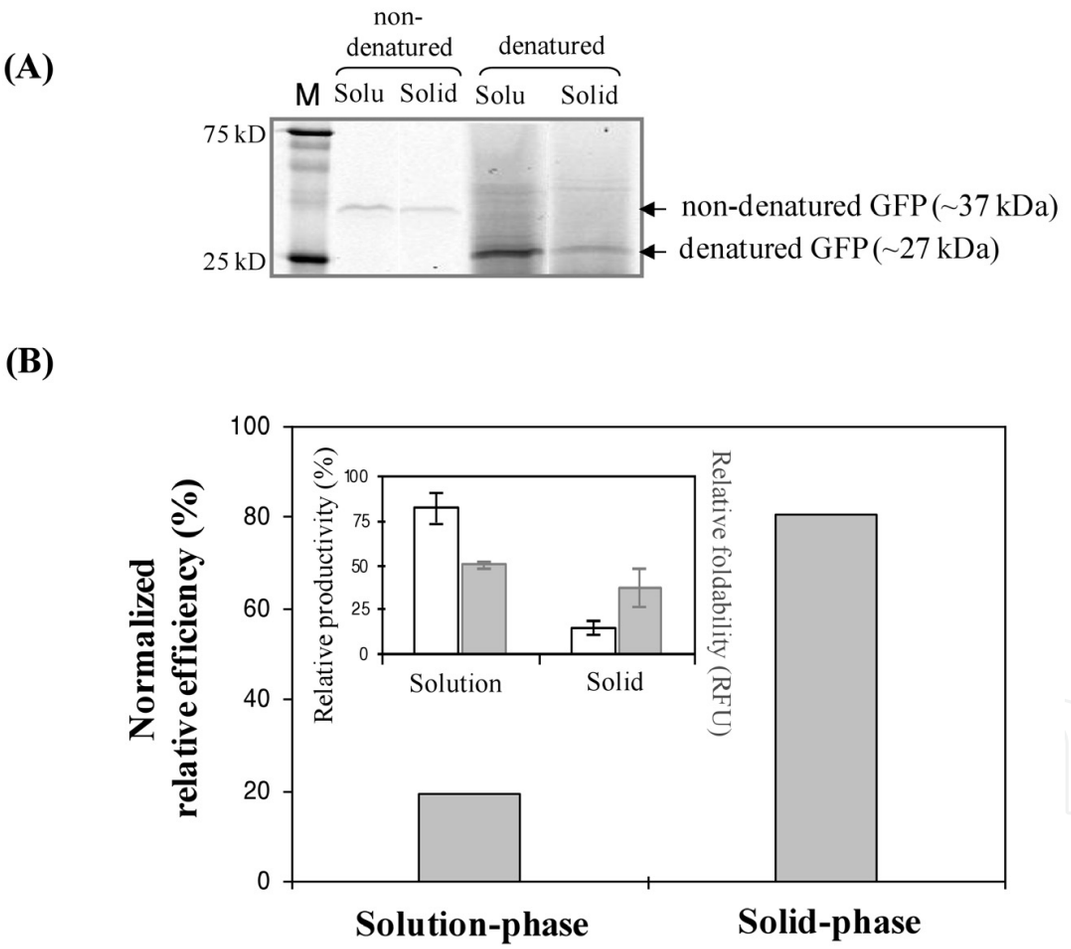

Figure 6. Comparison of the correct folding and productivity of GFP produced by solid-phase and solution-phase CFPS systems. (A) SDS-PAGE of non-denatured (folded) GFP (leftmost two lanes) and denatured GFP (rightmost two lanes). (b) Quantitative measurements of the relative efficiency of Solid versus Solution-phase in terms of ratio values were plotted after recombining the productivity (white column) and foldability (grey column) performance (shown in inset) from the solution or solid-phase systems. All plots and error bars represent average and standard coefficient values of more than four independent experiments. M, molecular weight markers.

6. Conclusion and future perspective

This chapter described a novel solid-phase cell-free translation system in which template molecules (DNAs/mRNAs) were captured onto solid-surfaces to simultaneously induce co-translational folding and synthesize proteins in a more native-state form. A newly constructed biotinylated linker-DNA is ligated to the 3′ ends of the mRNA molecules to attach the mRNA-template on a streptavidin-coated surface and further to enable the subsequent reactions of cell-free translation on surface. The protein products are therefore directly synthesized onto solid-surfaces and furthermore discovered to adopt a more native state with proper protein folding and enough biological activity compared with conventional solution-phase approaches. The approach described in this chapter may enables to embrace the concept of the transformation of 'DNA-to-Protein microarrays' using solid-phase cell-free protein synthesis system and thus to the development of high-throughput, CFPS platform to the field of functional proteomics.

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7. References

[1] Nirenberg MW, Matthaei JH. The dependence of cell-free protein synthesis in E. coli upon naturally occurring or synthetic polyribonucleotides. *Proc Natl Acad Sci U S A*. 1961, 47, 1588-1602.
[2] Ellis RJ. Macromolecular crowding: obvious but underappreciated. *Trends Biochem Sci*. 2001, 26, 597-604.
[3] Munishkina LA, Cooper EM, Uversky VN, Fink AL. The effect of macromolecular crowding on protein aggregation and amyloid fibril formation. *J Mol Recognit*. 2004, 17, 456-464.
[4] Ge X, Luo D, Xu J. Cell-free protein expression under macromolecular crowding conditions. *PLoS One* 2011, 6, e28707.
[5] Fedorov AN, Baldwin TO. Cotranslational protein folding. *J Biol Chem*. 1997, 272, 32715–32718.
[6] Komar AA. A pause for thought along the co-translational folding pathway. *Trends Biochem Sci* 2009, 34, 16–24.

[7] Kramer G, Boehringer D, Ban N, Bukau B. The ribosome as a platform for co-translational processing, folding and targeting of newly synthesized proteins. *Nat Struct Mol Biol.* 2009; 16:589–597.

[8] Han Y, David A, Liu B, Magadán JG, Bennink JR, Yewdell JW, Qian SB. Monitoring cotranslational protein folding in mammalian cells at codon resolution. *Proc Natl Acad Sci U S A*. 2012, 109, 12467-12472.

[9] Ugrinov KG, Clark PL. Cotranslational folding increases GFP folding yield. *Biophys J.* 2010, 98, 1312-1320.

[10] Liang, ST, Xu, YC, Dennis, P, Bremer, H. mRNA composition and control of bacterial gene expression. *J. Bacteriol.* 2000, 182, 3037–3044.

[11] Mathews, MB, Sonenberg, N, Hershey, JWB. Origins and principles of translational control. In *Translational Control of Gene Expression* (Sonenberg, N, Hershey, JWB, Mathews, MB, eds), 2000, 1–31, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[12] O'Brien EP, Vendruscolo M, Dobson CM. Prediction of variable translation rate effects on cotranslational protein folding. *Nat Commun.* 2012, 3, 868.

[13] Biyani M, Nishigaki K. Single-strand conformation polymorphism (SSCP) of oligodeoxyribonucleotides: an insight into solution structural dynamics of DNAs provided by gel electrophoresis and molecular dynamics simulations. *J Biochem.* 2005, 138, 363-73.

[14] Rambo RP, Tainer JA. Bridging the solution divide: comprehensive structural analyses of dynamic RNA, DNA, and protein assemblies by small-angle X-ray scattering. *Curr Opin Struct Biol.* 2010, 20, 128-137.

[15] Merrifield, RB. Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide. *Journal of the American Chemical Society.* 1963, 85, 2149-2154.

[16] Lockley AK, Jones CG, Bruce JS, Franklin SJ, Bardsley RG. Colorimetric detection of immobilised PCR products generated on a solid support. *Nucleic Acids Res.* 1997, 25, 1313-1314.

[17] Adessi C, Matton G, Ayala G, Turcatti G, Mermod JJ, Mayer P, Kawashima E. Solid phase DNA amplification: characterisation of primer attachment and amplification mechanisms. *Nucleic Acids Res.* 2000, 28, e87.

[18] Ståhl S, Odeberg J, Larsson M, Røsok O, Ree AH, Lundeberg J. Solid-phase differential display and bacterial expression systems in selection and functional analysis of cDNAs. *Methods Enzymol.* 1999, 303, 495-511.

[19] Marble HA, Davis RH. RNA transcription from immobilized DNA templates. *Biotechnol Prog.* 1995, 11, 393-396.

[20] Catt K, Niall HD, Tregear GW. Solid phase radioimmunoassay. *Nature.* 1967, 213, 825-827.

[21] Butler JE. Solid supports in enzyme-linked immunosorbent assay and other solid-phase immunoassays. *Methods.* 2000, 22, 4-23.

[22] Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science*. 1995, 270, 467-470.

[23] Schena M, Shalon D, Heller R, Chai A, Brown PO, Davis RW. Parallel human genome analysis: microarray-based expression monitoring of 1000 genes. *Proc Natl Acad Sci U S A*. 1996, 93, 10614-10619.

[24] Park N, Um SH, Funabashi H, Xu J, Luo D. A cell-free protein-producing gel. *Nat Mater.* 2009, 8, 432-437.

[25] Andrade JD, Hlady V. Protein adsorption and materials biocampatibility: a tutorial review and suggested hypotheses. In: Dusek K, editor. *Advances in polymer science*. Berlin: Springer, Heidelberg, 1986.

[26] Weetall HH. Preparation of immobilized proteins covalently coupled through silane coupling agents to inorganic supports. *Appl Biochem Biotechnol.* 1993, 41, 157-188.

[27] Laitinen OH, Nordlund HR, Hytönen VP, Kulomaa MS. Brave new (strept)avidins in biotechnology. *Trends Biotechnol.* 2007, 25, 269-77.

[28] Biyani M, Husimi Y, Nemoto N. Solid-phase translation and RNA-protein fusion: a novel approach for folding quality control and direct immobilization of proteins using anchored mRNA. *Nucleic Acids Res.* 2006, 34, e140.