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REVIEW

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Developing cell-free biology for industrial applications

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Abstract Although cell-free protein synthesis has been practiced for decades as a research tool, only recently have advances suggested its feasibility for commercial protein production. This focused review, based on the 2005 Amgen Award lecture, summarizes the relevant progress from the Swartz laboratory. When our program began, projected costs were much too high, proteins with disulfide bonds could not be folded effectively, and no economical scale-up technologies were available. By focusing on basic biochemical reactions and by controlling cell-free metabolism, these limitations have been methodically addressed. Amino acid supply has been stabilized and central metabolism activated to dramatically reduce substrate costs. Control of the sulfhydral redox potential has been gained and a robust disulfide isomerase added to facilitate oxidative protein folding. Finally, simple scale-up technologies have been developed. These advances not only suggest production feasibility for pharmaceutical proteins, they also provide enabling technology for producing patient-specific vaccines, for evolving new enzymes to enable biological hydrogen production from sunlight, and for developing new and highly effective water filters. Although many challenges remain, this newly expanded ability to activate and control protein production holds much promise for both research and commercial applications.

Keywords Cell-free biology · Cell-free protein synthesis · Metabolic engineering · Cytomim · Oxidative protein folding · Cell-free scale-up

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Introduction

Cell-free protein synthesis is a concept that is decades old, and, in fact, was used to help decipher the genetic code 45 years ago [14]. However, until recently, this approach has remained a relatively expensive laboratory technique. Yet with the wealth of techniques and knowledge now available in fields such as molecular biology, metabolic engineering, and biochemical engineering, we are reassessing the potential of cell-free biology. The exciting image that emerges has motivated many researchers to rationally analyze and redesign cell-free systems. These include, among others: Professor Spirin, Professor Choi, researchers at Roche Diagnostics and Invitrogen, Professor Yaeta Endo, Professor Takuya Ueda, Professors Yokoyama and Kigawa, and Professors Yamane and Nakano. With due respect to their many advances, this report is based on the Amgen Lecture presented at the 2005 national meeting of the Society for Industrial Microbiology. Consequently, it will focus primarily on *Escherichia coli* system developments emerging from my laboratory at Stanford University.

The fundamental goal for our research has been to recognize and control the chemical reactions affecting cell-free system performance. Protein production is central to our program, and Fig. 1 presents a top level analysis. We seek to guide a polymerization and folding reaction that requires monomers (amino acids), a source of energy, and a source of information to empower and guide specific catalysts to synthesize our product, a complex protein. Especially because industrial applications are targeted, it has also proven essential to provide sufficient understanding of the system so that these complicated biochemical transformations can be performed efficiently and with confidence in large scale industrial equipment.

What is cell-free biology? Why use it?

Cell-free protein synthesis is part of a more general pursuit entitled Cell-free Biology and defined as "the

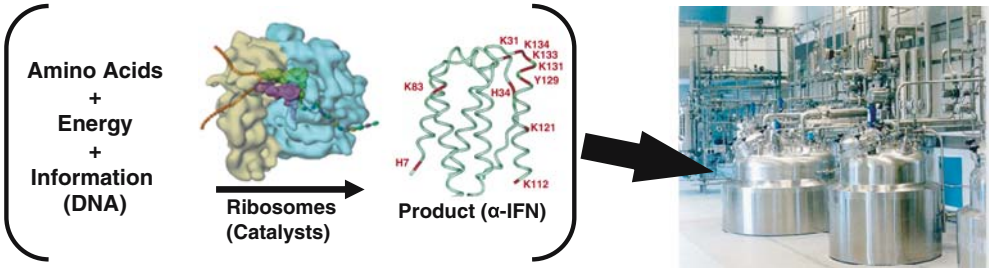

Fig. 1 The fundamentals of industrial cell-free protein synthesis

reproduction, study, and exploitation of complex biological processes WITHOUT intact cells". As suggested by Fig. 2, we then gain access to the inner workings of the cell. We can activate, integrate, and focus metabolism on our single objective without the need to protect the cell's DNA heritage. There is no longer a need (or the ability) for the cell to adjust its catalytic composition in response to challenges from the external environment. Furthermore, the absence of a cell wall avoids transport limitations and allows direct access for substrate additions, product removal, environmental monitoring, and rapid sampling. Transitioning to cell-free biology is a little like leaving home for the first time. Losing the protective support structure of a living, intact cell is unnerving, but the new opportunities are very exciting.

Figure 3 illustrates the basics of cell-free protein synthesis as embodied by the approach to be discussed in this report, combined transcription and translation using *E. coli* cell extracts. The extract provides the catalysts and other recycled helpers such as tRNAs and translation factors. Since protein synthesis is the most energy intensive process in living cells, a durable and inexpensive energy source is an absolute requirement. Phosphoenol pyruvate is a traditional energy source and regenerates ATP by transfer of a phosphate bond. In addition, the primary substrates, amino acids and nucleotides (NTPs), must be available at adequate concentrations.

If these requirements are satisfied and the typical T7 promoter and T7 RNA polymerase are used, the cell-free system allows the channeling of all metabolic resources to the synthesis of a single protein. In combination with the open nature of the system, this attribute allows unprecedented control of metabolism in its broadest sense to facilitate the synthesis and folding of the desired product. Being released from the need to maintain viability provides great freedom in process design and control and also enables the production of toxic proteins. The short duration and high productivity of batch reactions translate into very high overall productivity and low projected capital costs. Finally, the versatility of the system has enabled the pursuit of many applications. In my laboratory alone, we are pursuing: the production of protein pharmaceuticals and patient-specific vaccines, the evolution of new enzymes for producing hydrogen, the

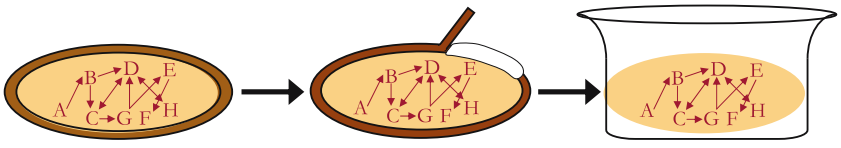

Fig. 2 Accessing the inner workings of the cell. Cell-free biology seeks to reproduce complex biological processes without a living cell, and, in fact, without a cell wall

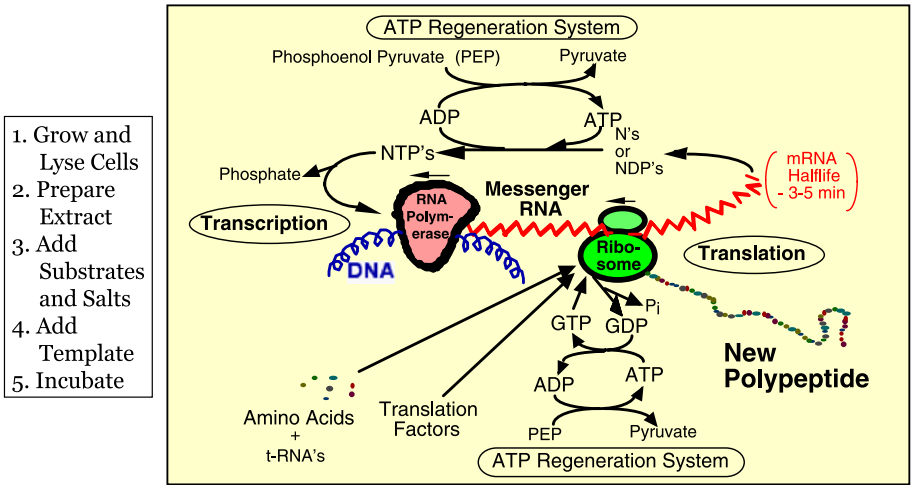

Fig. 3 The basics of cell-free protein synthesis. For this combined transcription and translation *E. coli*-based system, cell extract is prepared by growing cells at high growth rate, washing away the spent medium, breaking the cells by high pressure homogenization, and removing the cell wall and much of the chromosomal DNA by centrifugation. Substrates, salts, and a DNA template are then added to activate mRNA and protein synthesis; both of which are empowered by a durable energy source

development of novel water filters, and high throughput functional genomics.

What were the challenges and how did we approach them?

In spite of the exciting projected advantages, our early program faced many challenges. Product yields were low, expenses were very high, it was difficult to fold complex proteins (especially those with disulfide bonds), and no viable scale-up methods existed. It was truly a technology limited to small scale research applications.

Faced with these challenges, it was easy to be intimidated by the complexity of the system and by the further challenges involved in seeking to reproduce complex biological processes without the support of a living cell. We approached these challenges guided by three principles: (1) we would use industrial process cost models to identify the most advantageous improvement strategies, (2) we would focus on individual (bio)chemical reactions and pathways, and (3) most importantly, we would seek to activate authentic biological processes to take full advantage of *E. coli*'s prodigious metabolic capabilities. The highly evolved robustness and efficiencies of natural biology would provide our role models.

The first breakthrough

The simplest (and most superficial) analysis of the early system contemplated the challenge as the control and support of two chemical reactions:

1. Amino acids + ATP→ PROTEIN + ADP + Phosphate
2. Phosphoenol pyruvate + ADP → ATP + Pyruvate

The obvious focus is on the product, but it turned out to be more important to focus on the substrates. Early work showed that a reaction mixture incubated without a DNA template lost its ability to produce protein, even though no transcription or translation had occurred. Activity could only be restored by adding both fresh energy source (PEP) and fresh amino acids. An assessment of amino acid concentrations then revealed the disappearance of arginine, tryptophan, and cysteine. (Later work would also show that serine is also depleted.) Much more important, though, was the observation that alanine, aspartic acid, and asparagine were being synthesized to high concentrations. Central metabolism was active! In retrospect, this may seem a trivial realization, but, in fact, it had not been widely appreciated. This new way of thinking opened the way for all of the following development. The cell extract and cell-free reaction no longer constituted a complex, inscrutable "black box". Instead, the system was transformed into a collection of chemical reactions that could be understood and controlled.

The PANOx system

Guided by this revelation, the PANOx system was developed [7]. The acronym stands for **P**hosphoenol pyruvate (PEP), **A**mino acids, **N**AD$^+$, and **Ox**alic acid, and the cell-free metabolism is illustrated in Fig. 4. The observed accumulation of alanine was not a big surprise since pyruvate is a major byproduct of the energy producing reaction, and a single enzymatic conversion produces alanine from pyruvate. However, the accumulation of aspartic acid (asp) and asparagine (asn) was much more interesting. These compounds are produced from oxaloacetate, an intermediate of the TCA cycle.

Although oxaloacetate is also produced directly from PEP, cell-free reactions with only pyruvate additions still produced asp and asn. For this to occur, PEP synthetase (Pps) must first convert pyruvate into PEP at the cost of two phosphate bonds since ATP must be coincidentally converted to AMP. Thus, this pathway was robbing our ATP and almost certainly lowering protein synthesis yields. Fortunately, a previous, detailed study of Pps had revealed its inhibition by oxalic acid [13]. Accordingly, oxalic acid additions increased cell-free yields [6].

Since amino acids were being depleted, the initial concentration of each was simply raised from 0.5 to 2 mM. As the last modification, since Professor Spirin's lab had shown that acetyl phosphate (AcP) could be used as an energy source [16], we activated the pathway from pyruvate to AcP by adding the cofactors NAD$^+$ and CoenzymeA. The result of these combined modifications was an increase in the yield of chloramphenicol acetyl transferase (CAT) from 160 to 350 μg/ml. Later work with improved cell extracts would consistently demonstrate longer synthesis durations and produce yields of 600–700 μg/ml using the same insights. The PANOx technology was licensed to Roche Diagnostics and provided the basis for their high yielding cell-free protein synthesis kits.

But the amino acids were still not stable

As mentioned, early evaluation of system kinetics revealed the depletion of several amino acids. Arginine and cysteine, in particular, were exhausted in the first 30 min of the reaction. In later reaction formats, serine was depleted even faster. Yet only a small fraction of the initially added amino acids was being used for protein synthesis. The remainder was, from our point of view, being degraded. Tryptophan, although more stable than the other three was also potentially threatened by tryptophanase activity.

For the PANOx system, we had focused on adjusting the chemical composition of the reaction mixture to either activate or inhibit important reactions, and for tryptophan stabilization, we could simply grow our cells with a glucose-containing medium to avoid typtophanase expression. However, for the other three unstable amino acids,

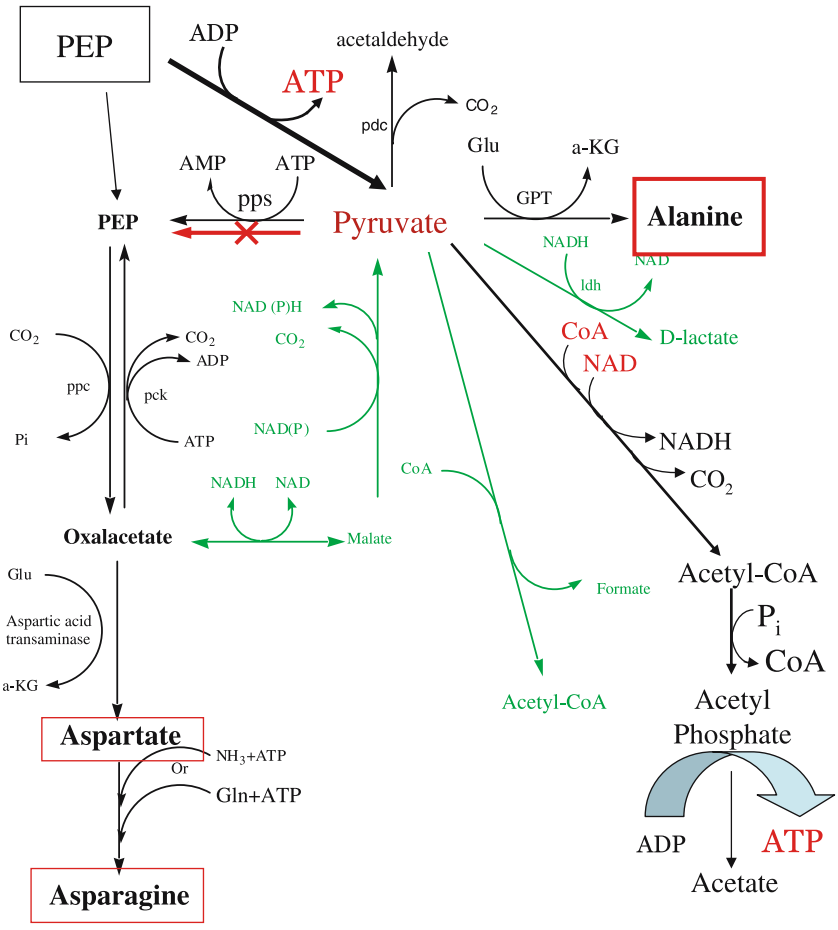

Fig. 4 Engineering cell-free metabolism. This diagram illustrates the metabolism associated with the PANOx cell-free system. Phosphoenol pyruvate is the energy source. Oxalic acid inhibits the energy wasting reverse reaction of pyruvate being converted to back to PEP. Increasing amino acid concentrations to 2 mM delays their exhaustion, and the addition of NAD^+ (and CoenzymeA) activates the conversion of pyruvate to acetyl phosphate as an additional source of ATP

more dramatic measures were needed. We still needed to identify the reaction(s) responsible for the depletion of each amino acid, but it was unlikely that we could find a specific inhibitor for each reaction that would not be detrimental to protein synthesis. Instead, the approach was first to identify an enzyme whose inactivation would: (1) stop the amino acid degradation, but (2) not affect the growth of the organism for cell extract preparation. Next, the gene that encodes that enzyme would be deleted.

For arginine, the targeted enzyme was arginine decarboxylase, the product of the *speA* gene [11]. It is the first enzyme on the committed pathway from arginine toward the polycations, putrescine and spermidine. These are essential compounds for cell growth, but they can also be derived from ornithine. This secondary pathway is sufficient as long as excess arginine is not present to inhibit it. Fortunately, it was known that spermidine inhibits arginine decarboxylase, and we could demonstrate that spermidine additions stabilized arginine. As shown in Fig. 5a, cell extract derived from the new *speA* mutant no longer degraded arginine. In a similar manner, two serine deaminases were avoided by a double *sdaAsdaB* mutant that produced cell extract with stable serine (Fig. 5b). The pathway toward glutathione synthesis had been proposed as the main cause of cysteine disappearance by Kim and Choi [5], and this was confirmed by radioactive tracer studies [11]. We therefore deleted the *gshA* gene that encodes glutamate cysteine ligase, the first enzyme in the glutathione synthesis pathway. As Fig. 5c indicates, this change stopped cysteine degradation during the cell-free synthesis reaction. Finally, the *tnaA* gene that encodes tryptophanase was also deleted to ensure tryptophan stability. Fortunately, even the combination of these five genetic modifications at the heart of central metabolism had no impact on the growth of the mutated organism. More importantly, the new cell extracts produced more product [11].

And the energy costs were much too high

From the very beginning of our research program we used SuperPro Designer™ from Intelligen, Inc. (Scotch Plains, NJ) to evaluate projected product costs. Even with the higher yields and stable amino acids, the costs were still much too high. Since they were dominated by the energy (PEP) and nucleotide costs (nucleoside triphosphates, NTPs), these substrates became the dominant focus of the research program. In fact, they are closely linked. Nucleoside monophosphates (NMPs) are much less expensive than the NTPs and can potentially be converted to the NTPs in situ if sufficient energy (ATP) can be supplied.

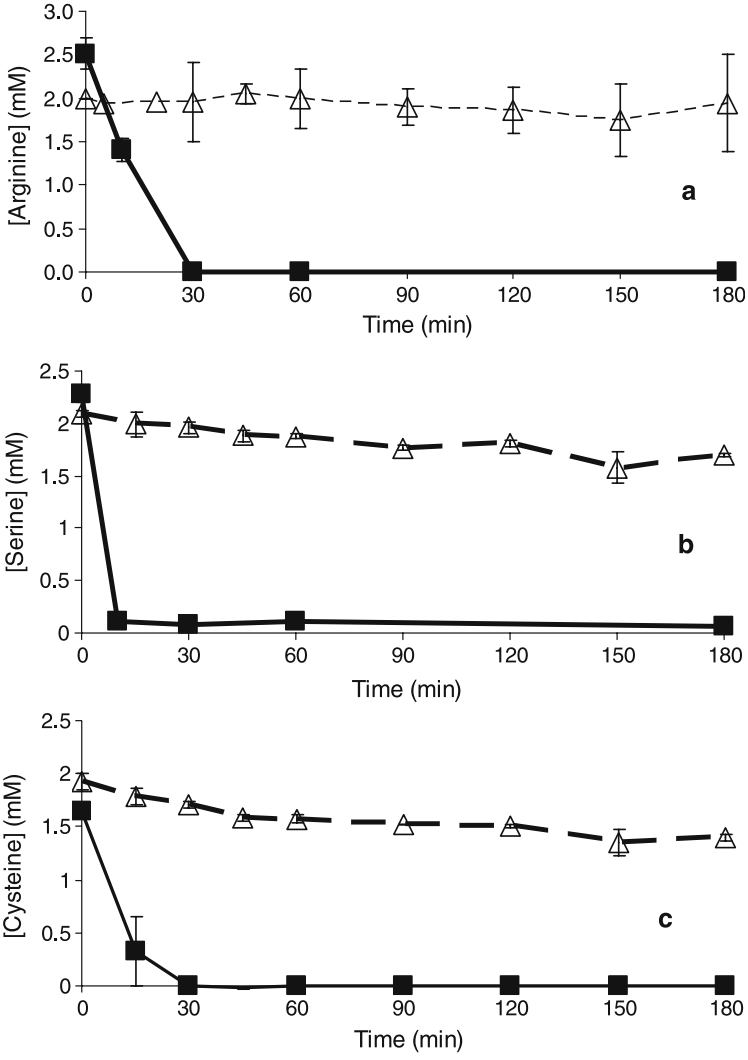

Fig. 5 Stabilizing amino acids. Comparison of wild type phenotype (*squares*) to mutated phenotype (*triangles*) for **a** the *speA* mutation that stabilizes arginine, **b** the *sdaAsdaB* double mutation that stabilizes serine, and **c** the *gshA* mutation that stabilizes cysteine

The most effective biological energy generator is oxidative phosphorylation (respiration). Although we hoped to activate this metabolic process in early experiments, it was clear that oxygen availability had no effect on product yields. We therefore adopted the hypothesis that a more natural chemical environment would encourage more natural metabolism. Since the cells are lysed by a very high shear rate event (with an Avestin continuous homogenizer operated at ≈18,000 psig), there was reason to believe that inverted inner membrane vesicles would be generated, and that these could be activated to produce ATP from reducing equivalents (NADH, primarily). The NADH would be produced by activated central metabolism and would be directly accessible to the NADH dehydrogenases on the external surface of the inverted vesicles. Michael Jewett therefore began a careful redesign of the cell-free chemical environment. As shown in Table 1, he found that he could replace the polyethylene glycol (PEG) with spermidine and putrescine (most likely to stabilize nucleic acids), remove the unnatural pH buffer, and reduce the concentrations of other key ions to cytoplasmic levels. Since the objective was to reduce costs, he then tested the new formulation with sodium pyruvate as the energy source. The results were dramatic [4]. Yields of CAT increased from less than 100 to over 700 μg/ml with this inexpensive energy source. These high yields were then shown to be dependent on the presence of oxygen, suggesting that, in fact, oxidative phosphorylation had been activated. This new system was called the Cytomim system, short for **cyto**plasmic **mim**ic. In addition to the cost savings, the use of non-phosphorylated energy sources avoids the accumulation of inhibitory phosphate concentrations and permits longer duration reactions.

Kara Calhoun then showed that with a bis–tris pH buffer and phosphate addition, glucose could be used as an even less expensive energy source [2]. Furthermore, relatively high protein yields were still obtained when glucose was the energy source and NMPs were substituted for NTPs [1]. When other expensive co-factors were also omitted, the protein yield per dollar of reagent costs increased by nearly 30-fold. At this point, reagents costs were no longer a barrier to commercial applications.

Table 1 The intracellular chemical environment provides the guidance for a new cell-free system, the "Cytomim" system (for **cyto**plasmic **mim**ic)

	Old (PANOx) system	*E. coli* cytoplasm	Cytomim system
PEG (% w/v)	2	0	0
HEPES Buffer (mM)	57	0	0
Putrescine (mM)	0	~20	1
Spermidine (mM)	0	~6	1.5
Acetate (mM)	135	Low (<20)	23
Ammonium (mM)	80	5–10	10
Phosphate (mM)	~30	~5	~5
Magnesium (mM)	23.3	~25 (~3 Free)	11.3
Energy source	Phosphoenol pyruvate	Oxidative phosphorylation	Oxidative phosphorylation

Subsequent work has since shown that glutamate is also an effective energy source, and that oxygen supply becomes a serious design constraint for the new systems. The latter will be further addressed in the scale-up section. Further results showed that ATP concentrations were dependent on the rate of oxygen transfer. This observation accentuated the question of what ATP concentrations were necessary for rapid protein synthesis. Unpublished results from our lab suggest protein synthesis k_ms for ATP and GTP of 27 and 14 μM, respectively. Fortunately, these are significantly lower than the ATP and GTP concentrations measured during the cell-free reactions. Energy supply is not limiting protein synthesis rates even when NMPs are used.

We still needed to improve protein folding!

The folding of most cytoplasmic proteins proceeds relatively efficiently in the Cytomim system. Even though CAT must form a homotrimer to be active, typically 70–80% of the expected enzyme activity is achieved as judged by total synthesis and by the published specific activity. Even proteins such as thioredoxin reductase and glutathione reductase are almost completely activated even though they must dimerize and an FAD cofactor must be installed in each monomer [9]. Yet, the first attempt to produce an active disulfide bond-containing enzyme failed miserably [8]. The product was the protease portion of murine urokinase requiring 6 disulfide bonds, and a relatively oxidized glutathione redox buffer was used in an attempt to drive the formation of new disulfide bonds. In some cases, a small amount of enzyme activity was initially produced, but it would then disappear. It turned out that the cell-free extract was actively reducing the system. Presumably, NADPH was being produced to provide reducing equivalents for disulfide bond reduction, and thioredoxin reductase and glutathione reductase were serving as the conduits for electron transfer. Since both enzymes require free –SH groups in their active sites, the cell extract was pretreated with iodoacetamide (IAM, 0.5–1 mM) before use. These pretreated extracts allowed the stable formation of disulfide bonds [8, 22], presumably because the reductases had been inactivated.

Figure 6 shows the effects of these modifications on the formation of soluble murine GMCSF (granulocyte macrophage colony stimulating factor) [20]. Even though GMCSF only requires two disulfide bonds, several system modifications are required to form significant soluble yields. First, the system –SH/S-S redox potential is stabilized by IAM pretreatment. Next, a GSSG/GSH redox buffer is used to provide a relative oxidizing environment. Finally, it is necessary to add the *E. coli* disulfide isomerase, DsbC, to catalyze disulfide exchange. It then turns out that most of the product accumulates in a soluble form, and that soluble product is fully bioactive [20]. It is now interesting to reflect on the generality of this approach.

We have successfully produced a number of disulfide-bond containing proteins. It was initially proposed that the fast translation rate in *E. coli* would not be compatible with the folding of mammalian proteins. The rate is about 16 amino acids per second in *E. coli* relative to a rate of 4–5 amino acids per second in mammalian cells. However, a careful measurement of the translation elongation rate in the *E. coli* cell-free system suggests an elongation rate of only 1.5–2 amino acids per second [18]. It is actually slower than for mammalian cells. It was also possible that the chaperone function in prokaryotic systems would not effectively assist the folding of eukaryotic proteins. Our results suggest that this is not true for most eukaryotic proteins. Nonetheless, although several observations suggest that the more natural chemical environment of the Cytomim system is beneficial for protein folding, more work is needed before we will know if providing eukaryotic chaperones will provide further benefits.

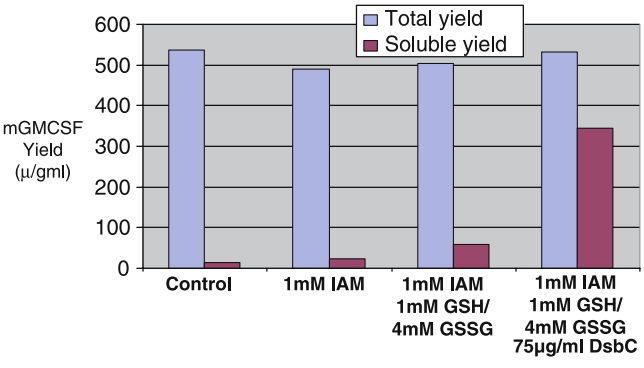

Fig. 6 Reengineering for co-translational oxidative protein folding. To achieve proper folding of murine granulocyte macrophage colony stimulating factor (mGMCSF), it was necessary to first stabilize the –SH/S-S redox potential, to then provide a relatively oxidizing environment, and finally to provide DsbC as a disulfide isomerase. A combination of all three measures was required

And now we have produced cell-free biology

As Fig. 7 illustrates, we have now produced a system that is much more than a transcription/translation system. We have truly activated a complex biological process without using living cells. The system depicted uses glutamate as an energy source to produce reducing equivalents from the TCA cycle. These reducing equivalents then fuel oxidative phosphorylation in which oxygen serves as the electron acceptor and membrane dependent respiration provides a plentiful ATP source. This, in turn, allows the use of NMPs. To improve efficiency and protein yields, we have stabilized amino acids through a series of five mutations. We have also modified the cell extract source genome to stabilize linear DNA templates [12]. Finally, a key "housekeeping" metabolic pathway has been inactivated (the reduction of disulfide bonds), the –SH/S-S redox level modified, and the DsbC levels augmented to allow the oxidative folding of secreted mammalian proteins. Most notably, with the last change we have passed beyond the realm of mimicking natural metabolism. In nature, oxidative protein folding is conducted in a separate compartment; distinct from the location of the transcription/translation reactions. With cell-free biology, we can efficiently conduct both polypeptide synthesis and oxidative folding in the same compartment.

For commercialization, we still needed to scale-up

At a minimum, we need to scale-up the production of the cell extract as well as the cell-free protein synthesis reaction. As might be expected, cells that are grown rapidly make more active cell extracts. We have therefore developed a fermentation medium and feeding protocol to enable growth to 40 optical density units at high growth rate [23]. This protocol allows the use of a defined medium for process consistency without diminishing extract performance. Furthermore, a careful analysis of the extract preparation procedure allowed the cost and duration of this process to be substantially reduced [10]. It is particularly interesting that an 80 min incubation without the addition of an energy source is just as effective at activating the cell extract as the traditional "run-off" reaction to which expensive energy sources were added. Although the ribosomes are still in the 70 S form after the less expensive incubation, the extract is just as active as when the ribosomes separate into subunits with the addition of an energy source.

Scaling up the cell-free reaction was a more significant challenge, especially with the need to provide oxygen. Notably, Alexei Voloshin found that the cell-free reaction works well when the reaction mixture is allowed to spread as a "big drop" in a thin film format on hydrophobic surfaces [19]. For example, the thin film reactions typically produce higher protein yields in this format than in the normal 15 µl volume used for development experiments. Figure 8 presents total and soluble yields for one of the more challenging product candidates, a fusion protein consisting of murine GMCSF and a scFv antibody fragment from a lymphoma surface receptor. In this case, the thin film format produces yields equivalent to those of the 15 µl reaction and significantly better than yields from a 500 µl test tube reaction. The last column indicates that a bubble column format also produces high yields. In this format, it is necessary to add a standard antifoaming agent to avoid foaming the reaction solution out of the tube. Surprisingly, the antifoaming agent does not seriously hinder protein expression or folding.

These principles were then extended into stirred tank reactor formats, most notably for the production of human insulin-like growth factor I (IGF-I). IGF-I solubility and activity requires the formation of three disulfide bonds even though it is a rather small protein (7,649 Da). In spite of many attempts, properly folded IGF-I could not be produced at any significant yield in *E. coli*. However, with the controlled –SH/S-S redox conditions and slower translation rates of the new cell-free technology, approximately 85% is soluble. Reactions at 2, 50 and 1,000 ml scales produced identical yields of 400 µg soluble IGF-I/ml. Reverse phase HPLC indicates that the soluble IGF-I is folded in the expected 2-to-1 ratio of properly folded to disulfide mismatched forms. Subsequent mass spectrometry analysis indicates that the product has the correct and uniform molecular weight of the formylated met-IGF-I. Subsequent work will now address the in situ removal of the formyl methionine N-terminus.

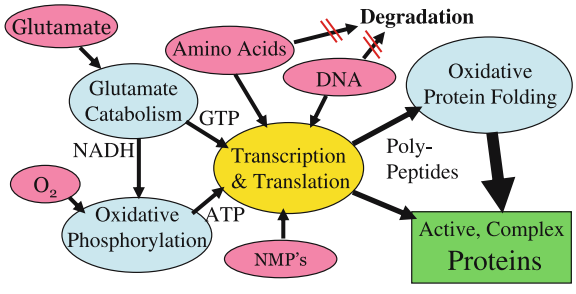

Fig. 7 This is Cell-Free Biology. As more metabolic systems become activated, cell-free protein synthesis becomes cell-free biology. A natural chemical environment activates glutamate catabolism and oxidative phosphorylation to provide an inexpensive and plentiful source of ATP and GTP. Now, relatively inexpensive nucleoside monophosphates (NMP's) can be used instead of nucleoside triphosphates. Genetic modifications to the cell extract source cells stabilize the amino acid substrates as well as linear DNA templates. Finally, control of the –SH/S-S redox potential activates oxidative protein folding for the economical production of secreted mammalian proteins

Now we can address a variety of exciting applications

Patient-specific protein pharmaceuticals

For many years, Professor Ron Levy and colleagues have been pursuing the treatment principle illustrated in

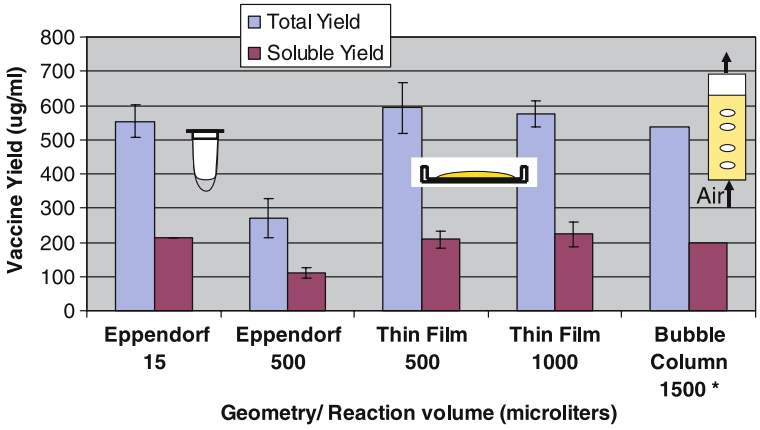

Fig. 8 New methods for cell-free scale-up. Scaling up the test tube reaction format reduces volumetric yields, but both the thin film and bubble column formats preserve the yields of both total and soluble product. In this case, the product is a GMCSF–scFv fusion protein vaccine candidate. The *asterisk* denotes that an antifoaming agent was required for stable bubble column operation

Fig. 9. B cell lymphoma is a cancer that occurs when a specific antibody-producing B cell loses control and divides with abnormally high frequency. The diseased cell still displays on its surface the antibody product unique to that cell, and this provides a specific point of attack. However, the immune system is unable to recognize this cell as abnormal, and the disease is not checked.

Animal and human studies have suggested that an appropriate vaccine can stimulate an immune response to that cell via its surface receptor antibody, and that this immune response can control the disease [3, 17]. These same studies have suggested that GMCSF is a potent fusion partner to stimulate the immune system for this purpose. Thus, the objective has been to produce such vaccines, and, of course, the challenge is that each patient requires a customized vaccine since the variable region of the surface receptor antibody is different for each patient. A biotechnology company, Genitope Corp., is now conducting a phase III clinical trial to evaluate this concept, but they use mammalian cells to produce full length antibodies which are activated as vaccines by covalent coupling of keyhole limpet hemacyanin. Human GMCSF is then co-administered. Although the approach is promising, the production process is long and expensive.

If lymphoma vaccines could be produced with cell-free technology using linear DNA templates, the process could go all the way from patient specimen to vaccine product without requiring living cells. This would allow the patient-specific vaccine production to be rapid and relatively inexpensive and would also reduce the risk of patient-to-patient cross-product contamination that might occur in a facility simultaneously producing many separate vaccines for many different patients. Recently, we have reported the cell-free production of a GMCSF–scFv fusion protein vaccine using the new cell-free technology [21]. Production has been scaled up using the thin film format, and initial mouse studies have indicated that this fusion protein format induces protective antibodies in mouse tumor models.

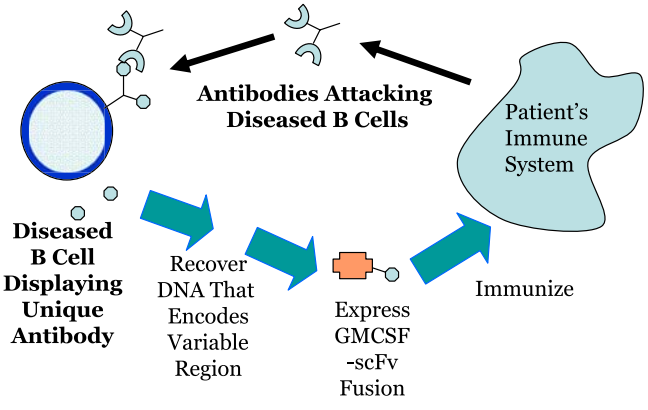

Fig. 9 Patient-specific lymphoma vaccines. A patient-specific immunotherapeutic proposal for treating B-cell lymphomas is shown here. The patient's own immune system is stimulated to attack the diseased B-cell. By fusing granulocyte macrophage colony stimulating factor (GMCSF) to the variable region of the diseased cell's surface receptor (an antibody), we hope to target the vaccine to dendritic cells to increase vaccine potency and specificity

Evolving a new hydrogenase for converting solar energy to hydrogen fuel

Nature provides countless examples of intriguing and potentially useful bioconversions. One of the more exciting is the conversion of sunlight into chemical energy. With the awareness of global warming and the impact of global energy economics, the possibility that we could convert solar energy into a portable fuel, hydrogen, is very exciting indeed. Figure 10 illustrates a new organism that might accomplish this objective. The photolysis center would capture the sunlight and use the energy to split water into protons, electrons, and molecular oxygen. The electrons would be captured by the electron carrying protein, ferredoxin, and would be transferred to a hydrogenase enzyme that would combine the electrons with the protons to make molecular hydrogen. The most obvious difficulty with this scheme is also illustrated. All known hydrogenases, especially the Fe–Fe hydrogenases with attractive reaction rates, are inactivated by molecular oxygen. Our objective then becomes the evolution of a new Fe–Fe hydrogenase that is tolerant to oxygen.

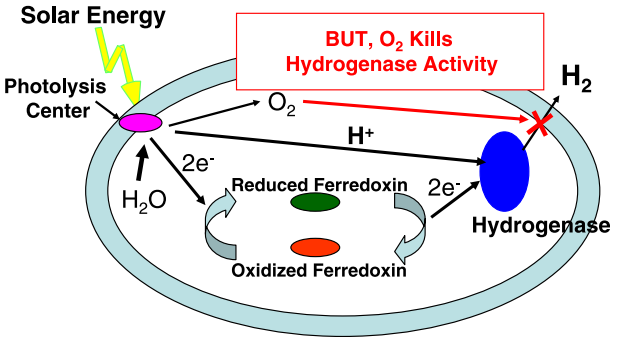

Fig. 10 Biological conversion of sunlight and water into HYDROGEN. Our objective is to engineer an organism that converts solar energy into hydrogen by splitting water. However, we first need to evolve an oxygen tolerant hydrogenase

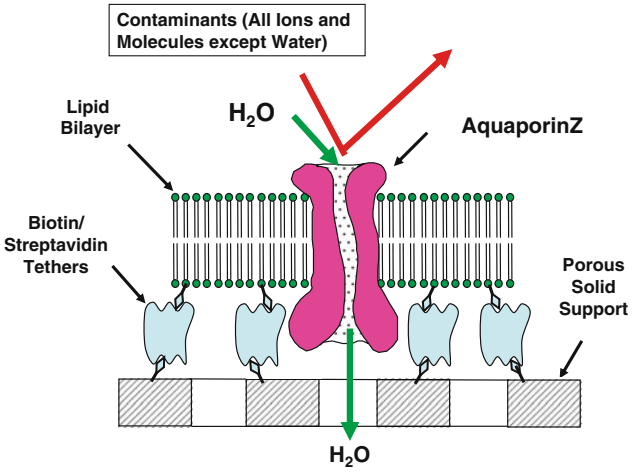

Fig.11 A new water filter based on biology. Our strategy is to exploit the specificity and high permeability of Aquaporin Z for water purification. Biotin/streptavidin coupling is used to attach a lipid bilayer to a porous support. Cell-free protein synthesis will be used to insert the Aquaporin Z into the membrane either before or after attachment

To accomplish this evolution, we are using cell-free synthesis to express libraries of mutated hydrogenase genes so that their products can be screened for hydrogenase activity and oxygen tolerance. The cell-free format allows a rapid screening for oxygen tolerance without requiring a gene cloning step or product purification. However, the major challenge was to express active hydrogenases in a cell-free format since the hydrogenase requires the assembly of a complex iron–sulfur center stabilized by cyanide and carbon monoxide. Fortunately, a breakthrough publication [15] recently identified helper proteins (and their genes) that assisted with the assembly of the *Chlamydomonas* Fe–Fe hydrogenase. We are now routinely producing active hydrogenase in the cell-free system and are beginning to screen for oxygen-tolerant variants.

Biological membranes as efficient water filters

The discovery of aquaporins as specific biological water channels warranted a Nobel Prize because of their potential importance in medicine, but it is just as intriguing to consider their use for water purification. Figure 11 indicates how this could happen. The objective is to tether a lipid bilayer membrane to a porous support and to insert aquaporin water channels into that membrane. Alternatively, the aquaporin could be installed into the membrane before its immobilization on the porous support. We have been able to produce high yields of active *E. coli* Aquaporin Z installed into lipid bilayer vesicles using our cell-free technology. We are now working to use a biotin/streptavidin tethering system to immobilize those membranes onto a regenerated cellulose ultrafilter to form a new, highly effective water filter.

Summary

By focusing on individual reactions and basic concepts, we have produced technology that is capable of relatively inexpensive production of complex proteins at large scale. This new capability presents exciting potential for the commercial production of protein pharmaceuticals, but also suggests the feasibility of patient-specific protein pharmaceuticals, protein evolution for biohydrogen applications, and the production of dramatically improved water filters. Because proteins and protein production are at the heart of biology, these new cell-free methods will open many new opportunities for applying biology to address pressing societal needs. We still do not know why the reaction slows and stops, and we have more to learn about folding complex proteins. We also have the challenge of producing proteins with post-translational modifications. Undoubtedly, many more challenges will be encountered. Still, we now have a very solid foundation on which to work. I am confident that the same fundamental approach that so far has been so productive will continue to yield exciting advances.

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