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Article

The Evolution of Cell Free Biomanufacturing

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Abstract: Cell free systems are a widely used research tool in systems and synthetic biology and a promising platform for manufacturing of proteins and chemicals. In the past, cell free biology was primarily used to better understand fundamental biochemical processes. Notably, *E. coli* cell free extracts were used in the 1960s to decipher the sequencing of the genetic code. Since then, the transcription and translation capabilities of cell free systems have been repeatedly optimized to improve energy efficiency and protein yield. Today, cell free systems, in combination with the rise of synthetic biology, have taken on a new role as a promising technology for just in time manufacturing of therapeutically important biologics and high-value small molecules. They have also been implemented in an industrial scale for the production of antibodies and cytokines. In this review, we discuss the evolution of cell free systems, advancements in cell free protein synthesis, and cell free metabolic engineering, and conclude with discussing the importance and feasibility of mathematical modeling in cell free systems.

Keywords: Cell free systems; Cell free protein synthesis; Synthetic biology

1. Introduction

Cell free biology is an emerging technology for research, and the point of care manufacturing of a wide array of macromolecular and small molecule products. A distinctive feature of cell free systems is the absence of cellular growth and maintenance, thereby allowing the direct allocation of carbon and energy resources toward a product of interest. Moreover, cell free systems are more amenable than living systems to observation and manipulation, hence allowing rapid tuning of reaction conditions. Recent advances in cell free extract preparation and energy regeneration mechanisms have increased the versatility and range of applications that can be produced cell free. Thus, the cell free platform has become a promising alternative to traditionally used living systems for biomanufacturing as well as biological research. In combination with the rise of synthetic biology, cell free systems today have not only taken on a new role as a promising technology for just in time manufacturing of therapeutically important biologics and high-value small molecules, but have also been utilized for applications such as biosensing, prototyping genetic parts, and metabolic engineering. They have also been used as educational tools at the high school and undergraduate levels for understanding synthetic biology due to their ease of use, rapid response times, and the availability of commercial kits for different cell free platforms including *E. coli*, Chinese Hamster Ovary (CHO), HeLa, and plant cells [1–3]. Thus, cell free technologies are promising tools that will likely be at the center of many future synthetic biology applications.

Arguably, today the most widely used cell free technology is cell free protein synthesis (CFPS), an *in vitro* platform for protein transcription (TX) and translation (TL). The role of CFPS in research is not new; cell free systems have been used for decades to explore fundamental biological mechanisms. For

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example, some of the first uses of CFPS were in the 1950s by Borsook [4] and Winnick [5] who used animal tissue homogenates to study how amino acids were incorporated into proteins. A few years later, *Staphylococcus aureus* extracts were used to confirm amino acid incorporation [6]. In 1956, the role of adenosine triphosphate (ATP) in protein production was discovered using rat liver extracts [7], and Nirenberg and Matthaei [8,9] demonstrated templated translation i.e., the now familiar codon code, using *E. coli* cell free extracts (this work later led to a Nobel Prize in 1968). What arguably could be recognized as the first precursor to modern cell free transcription and translation applications was developed in 1967 by Lederman and Zubay [10]; they developed a coupled transcription-translation bacterial extract that allowed DNA to be used as a template. Shortly after, Spirin and coworkers improved cell free extract protein production with a continuous exchange of reactants and products, allowing the system to run for tens of hours; however, these systems could only synthesize a single product and were energy limited [11]. More recently, energy efficiency of *E. coli* CFPS was improved by generating ATP with substrate level phosphorylation [12] and oxidative phosphorylation in the Cytomim system [13–15]. Since oxidative phosphorylation is a membrane associated process, the study of Swartz and colleagues revealed that membrane dependent energy metabolism can be activated in a cell free system, suggesting complex metabolism is still occurring. Swartz and colleagues have also explored the scalability of cell free systems, for example for the production of cytokines as well as antibodies in the industrial scale [16–18]. Another platform, myTXTL [19], uses a different metabolic process that couples ATP regeneration and inorganic phosphate recycling to extend the duration of protein production. Synthetic genetic circuitry can also be constructed to control gene expression using a variety of approaches. Bacteriophage RNA polymerases are commonly used in CFPS for transcription. However, the use of a vast array of bacterial regulatory elements based on the sigma factor family has recently been explored, allowing multi-layer genetic cascades to be easily implemented [19,20]. Lastly, cell free systems have also been used as educational tools, at the high school and undergraduate levels, for understanding synthetic biology owing to their ease of use and rapid response times [1,2]. Commercial kits are available for research and educational applications generated from different organisms, including *E. coli*, Chinese Hamster Ovary (CHO), HeLa, and plant cells [3]. Thus, the developments in CFPS have expanded its repertoire of applications, enabling it as a viable alternative to living systems for not only investigative research but also bioengineering and biomanufacturing on both small and large scales.

In this review, we discuss the evolution of cell free technologies, particularly, advancements in extract preparation, cell free protein synthesis and cell free metabolic engineering applications. We then conclude with a discussion of the mathematical modeling of cell free systems. Mathematical modeling of cell free processes could be critical to determining performance bottlenecks, and estimating the costs of cell free manufactured products.

2. Origin and preparation of cell free extracts

There are two broad classes of cell free systems: crude cell lysates and reconstituted systems. While crude extract-based systems, commonly prepared from *E. coli*, *S. cerevisiae*, rabbit reticulocytes, wheat germ and insect cells [21], consist of the biocatalysts remaining after cell lysis, reconstituted systems are well defined, prepared using only the factors essential for protein synthesis: purified enzymes, tRNAs, ribosomes, amino acids and energy molecules. The first purified extract of this kind, the Protein synthesis Using Recombinant Elements (PURE) system, was developed by Shimizu et al. in 2001 [22]. A similar system, based on *Thermus thermophilus*, was later developed by Zhou et al. [23]. Other specialized systems based on PURE have also been developed [24–27]. These specialized systems have been utilized in applications including the synthesis of disulfide-bonded functional aglycosylated Immunoglobulin G (IgG) and G-protein coupled receptors, and the study of the effects of liposomes on the solubility of aggregation-prone membrane proteins. Such reconstituted systems offer two main advantages over crude extracts. First, they are a valuable research tool for studying biological processes including protein expression and folding in the context of a completely defined reaction mixture. For

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example, given the precise knowledge and control of the components in the reconstituted system, it is possible to study the role of individual additions such as chaperones, translation elongation factors, ribosome release factors, and other molecules. Li et al., in a study analyzing the influence of such additions in the PURE system, showed that the efficiency of protein synthesis was limited by translation elongation capacity, ribosome release and ribosome recycling. When the authors changed the ratio of elongation factors, release factors and recycling factors to ribosome concentration to more closely resemble *in vivo* conditions, a 5-fold improvement in the yield of firefly luciferase reporter protein was observed [28]. The second advantage of reconstituted systems, such as the PURE system, is that they do not contain proteases and nucleases, further improving the production of many proteins [29]. Despite these advantages, reconstituted systems suffer from two major drawbacks: higher cost ($0.99/$\mu$L for a PURE reaction vs. $0.15–0.57/$\mu$L for a crude extract reaction; price for commercial kits [3]), and lower yields [30]. In this regard, crude cell extracts prevail; they are less expensive, especially for reactions carried out in larger scales [29]. They also offer more complex metabolic capabilities that can be exploited for energy regeneration, extending the duration of protein synthesis. Toward these advantages, the preparation of crude cell free extracts, which has undergone a significant evolution since the early applications in the 1950s and 1960s, is now an area of considerable focus.

Cell free extracts are commonly derived from crude cell extracts, where the cell's transcription and translation machinery is retained while cellular debris and chromosomal DNA are discarded (Fig. 1). Cells are typically grown until they reach exponential phase, when they are harvested and lysed commonly using a high pressure homogenizer or a specialized bead mill [31]. Early extracts were prepared by centrifugation of lysates at 30000×g followed by the addition of a mixture of amino acids, adenosine triphosphate (ATP) and other energy molecules, salts, and buffer [32,33]. In the early 2000s, several changes were made by different research groups to make the extract preparation protocol more efficient including centrifugation at a lower rate (12000×g), the use of shake flask fermentation, and the overexpression of the T7 RNA polymerase in the commercial BL21 (DE3) *E. coli* strain during extract preparation [34–36]. Alternatives to the high pressure homogenization step, which include the use of bead vortex mixing [31] or lytic enzymes [37], have also been recently explored. It is also possible to delete or overexpress certain genes in the source cell to yield customized cell free extracts. For example, the Swartz group made several gene deletions in *E. coli* A19 cells before harvesting and extract preparation, improving the protein yield in the extract by up to 250% [38,39]. More recently, a new extract design scheme was implemented by the Jewett lab to combinatorially mix different extracts, each containing a unique overexpressed enzyme, to construct a full biosynthetic pathway [40]. The same group also optimized the extract preparation procedure to better accommodate the use of genetic circuits [41]. These developments show that the extract preparation process can be modified depending on the end goal. However, there are still important unanswered questions in extract preparation. For instance, it still remains to be explored how one can selectively delete enzymes only in cell free extracts. Continued research in this area could pave the way for minimal extracts highly optimized for a known application.

3. Applications of cell free technologies

Cell free protein synthesis (CFPS) has been utilized in a wide range of applications from the production of pharmaceutical proteins [42–44] to the production of libraries for protein evolution and structural genomics [45]. Complex post-translational modifications that are typically difficult to carry out using bacterial extracts have also recently been achieved in cell free. For example, N-linked glycoproteins have been produced in an *E. coli*-based cell free extract with the addition of a purified oligosaccharyltransferase (OST) and its lipid-linked oligosaccharides (LLOs) [46]. A single pot glycoprotein synthesis system was also developed, potentially allowing for the production of personalized protein therapeutics [47]; in this system, the OSTs and LLOs were expressed in the *E. coli* host strain which resulted in glycosylation-competent lysates. Other specialized proteins have also been produced in cell free systems. The ability to add membrane mimics such as surfactants or

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liposomes to the extract as stability agents has allowed the production of membrane proteins [48–51]. Vaccines [44,52], protein assemblies [53–55], and proteins incorporating non-natural amino acids [56–58] have also been synthesized using CFPS systems. Disulfide-bonded proteins and antibodies have also been synthesized by adding components that facilitate the formation of these bonds to the mixture such as glutathione reductase, thioredoxin reductase, iodoacetamide, and disulfide isomerase (DsbC) [26,29,59,60]. Point-of-care protein manufacturing is also possible when microfluidic reactors are used. Compared to batch reactions, continuous flow microreactors typically offer users more precise control over mixing [61,62]. An automated on-chip CFPS reactor has been developed that runs transcription and translation reactions simultaneously but in separate compartments [63]. Each process can be optimized independently and the quasi-continuous supply of new mRNA from the TX chamber to the TL chamber allows a longer CFPS reaction and increases protein yield. Other microfluidic platforms integrate purification methods like dialysis and affinity chromatography [64,65]. For example, cecropin B, an antimicrobial peptide that is widely used to control biofilm-associated diseases, has been produced at a clinically relevant dose in a few hours using a microfluidic device with on-chip protein purification [65]. A continuous exchange microfluidic reactor using nanofabricated membrane to allow for extended reaction times and improved yields has also been developed with the goal of producing single dose therapeutic proteins at the point-of-care [66]. Moreover, cell free extracts can be lyophilized and stored at -80°C for more than a year without degradation [29]. They can then be rehydrated with water and then incubated using the body's heat to activate the extract components, highlighting the portability of the cell free platform [67]. The versatility of cell free systems is demonstrated in their uses beyond biomanufacturing.

Applications of cell free systems in synthetic biology are varied, from diagnostics to fundamental discovery and prototyping. Biosensing is an area where cell free systems have recently proven to be useful. They possess a unique advantage over whole cells because of their ability to detect species that are cytotoxic or impermeable to the cell wall [67]. These systems have been deployed to detect pathogens such as norovirus [68], Ebola virus [69] and Zika virus [70]. In addition, initial studies have showed that paper-based cell free sensors can detect the presence of heavy metals such as mercury and drugs such as γ-hydroxybutyrate, by utilizing the transcriptional regulators, MerR and BlcR, respectively [71]. The portability offered by these systems further underscores their usefulness in the field. CFPS has also been used in the development of minimal cells, the simplest cellular mimics that consist of only the genes essential for survival. Minimal cells are often described as biological analogs to the hydrogen atom which has served to uncover many fundamental phenomena in chemistry [72]. Their bottom-up construction has been made possible mainly by the advancements in two areas: compartmentalization strategies and programmable genetic circuits [19,73–76]. Toward this goal, proteins have been expressed in compartments such as liposomes [19,77], phospholipid vesicles [20,75] and hydrogel particles [78], and genetic circuits that encode oscillations [79,80], negative feedback loops [81], or riboswitches acting as regulatory elements [82] have been developed in CFPS systems. Interacting minimal cells have also been recently developed [83]. Cell free systems have also been used for prototyping novel genetic parts or circuits before using them *in vivo*. Prototyping can be done more efficiently in cell free systems because of the tighter control over plasmid dosage, inducer concentrations, pH, temperature and salt concentrations [67]. The ability to use linear PCR templates in cell free further accelerates this process [67]. Moreover, developments in experimental setup and analysis techniques such as the use of acoustic liquid-handling robots [84], real-time fluorescent reporters [85], microfluidics [86], and droplet-based expression [87] have allowed the prototyping to be carried out in high-throughput rates [67]. The relative ease of manipulating cell free systems makes them attractive tools for investigating complex processes.

Cell free systems have gained wide interest in metabolic engineering applications, primarily to circumvent the significant barriers in traditional *in vivo* processes [88]. Cell free metabolic engineering (CFME) has been practiced in both purified and crude cell extracts. For example, a major challenge in *in vivo* metabolic engineering is achieving high flux through synthetic pathways of interest. This is because

 doi:10.20944/preprints202003.0461.v1

the cells have their own objectives e.g., growth or maintenance, which drives metabolic flux away from desired pathways. The complexity of living cells also makes computational modeling and optimization of metabolic flux difficult [89]. Cell free systems, on the other hand, can be accurately modeled and the reaction environment tuned according to the bio-synthetic needs. Cell free systems offer many advantages for the study, manipulation and modeling of metabolism. Central amongst these is direct access to metabolites and the biosynthetic machinery without the interference of a cell wall. This allows interrogation of the chemical microenvironment while the biosynthetic machinery is operating, potentially at a fine time resolution. Eliminating the need to maintain cell viability also allows the full allocation of energy resources to the production of products of interest. Taken together, these characteristics make metabolic engineering more amenable in cell free systems. For example, important chemicals such as ethanol [90], n-Butanol [91], and ethyl(S)-2-ethoxy-3-(p-methoxyphenyl)propanoate (EEHP) [92] have all been produced cell free. The conversion of glucose to ethanol by using six purified enzymes by Guterl et al. [90] was a notable achievement in the field. Zhu et al. demonstrated the production of an aerobic enzymatic fuel cell from glucose [93]. The need to manage the flux of reducing equivalents was addressed by the design of a molecular purge valve utilizing NoxE, pyruvate dehydrogenase, and its mutated form [94]. This valve was utilized in the production of isoprene [94]. Protein synthesis has also been improved using CFME approaches; Calhoun and Swartz [95], for example, performed chromsosomal deletions in the source cells to address the problem of cell free amino acid degradation. Gene overexpression approaches have also been used to improve protein yields [96,97]. CFME has also been used to address certain bottlenecks in CFPS such as the need for energy and cofactor regeneration in cell extracts. One of the early examples involved adding oxalate, coenzyme A and NAD+ to inhibit a futile cycle while producing ATP from pyruvate [98]. More involved metabolic pathways, including the activation of glycolysis and oxidative phosphorylation, have been utilized in different extracts [13,95,99]. These efforts have paved the way for the use of cheaper energy sources (glucose, pyruvate or glutamate) and nucleotides (NMP) as well as increased the duration of the protein synthesis reactions. As a testament to the potential of CFME, a novel pathway termed non-oxidative glycolysis has been designed, enabling 100% conservation of carbon in sugar catabolism to acetyl-CoA [100]. However, despite these achievements and the advantages of cell free over *in vivo* processes, a fundamental challenge remains: the identification of genetic manipulations that most effectively accomplish a desired phenotypic goal [101]. Due to the complexity and immense interconnectivity of metabolic networks, even for simple prokaryotic organisms like *E. coli*, making the appropriate genetic manipulation for a desired function is not intuitive. To this end, the systems-level analyses offered by various mathematical models can prove indispensable for the researcher to make an informed decision.

4. Mathematical modeling of cell free systems

If cell free systems are to become a mainstream technology for advanced applications such as point of care therapeutic manufacturing [102], we must first understand the performance limits and costs of these systems [15]. A critical tool towards this goal is mathematical modeling. Decades before the genomics revolution, mechanistically structured *in vivo* metabolic models arose from the desire to predict microbial phenotypes resulting from changes in intracellular or extracellular states [103]. The single cell *E. coli* models of Shuler and coworkers pioneered the construction of large-scale, dynamic metabolic models that incorporated multiple regulated catabolic and anabolic pathways constrained by experimentally determined kinetic parameters [104]. Shuler and coworkers generated many single cell kinetic models, including single cell models of eukaryotes [105,106], minimal cell architectures [107], and DNA sequence based whole-cell models of *E. coli* [108]. As biological understanding grew, metabolic models became more sophisticated, able to describe cellular processes such as RNA synthesis, chromosome synthesis, regulated catabolic and macromolecular synthesis pathways using ordinary differential equations [109]. Karr et al. (2012) have developed a whole cell model of *Mycoplasma genitalium*, accounting for all genes and their interactions in the cell [110]. The model is constructed

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with independent sub-models describing different components of the cell, which is able to describe the life cycle from the level of single molecules. Each sub-model was parameterized and tested independently, thus it is possible that this whole cell model will not hold true under all conditions for the specified parameters. Even though some of these models have been successful, their formulation is complex, nonlinear and requires a large set of parameters that are computationally expensive to estimate. To overcome such obstacles, constraint based methods [111] have been developed to help describe biochemical networks without the need of kinetic parameters of the cellular processes. Stoichiometric reconstructions of microbial metabolism, popularized by constraint based approaches such as flux balance analysis (FBA), have become standard tools to interrogate metabolism [112]. FBA and metabolic flux analysis (MFA) [113], as well as convex network decomposition approaches such as elementary modes [114] and extreme pathways [115], model intracellular metabolism using the biochemical stoichiometry and other constraints such as thermodynamic feasibility [116,117] under pseudo steady state conditions. Constraint based approaches use linear programming [118] to predict productivity [111,119], yield [111], mutant behavior [120], and growth phenotypes [121] for biochemical networks of varying complexity, including genome scale networks. Constraint based models have also been used to identify strategies for the overproduction of desired compounds. These strategies include genetic knockouts or the addition of heterologous enzyme pathways to an organism's metabolic network and have been used in developing useful bacterial strains for the production of biofuels [122], high-value chemicals [123–125] and pharmaceuticals [126,127]. Stoichiometric reconstructions have been expanded to include the metabolic demands for protein synthesis based on the DNA and protein sequences, where the transcription and translation processes have been integrated into metabolism [128]. Since then, these models have been expanded into genome-scale with detailed descriptions of gene expression (ME-Model) [112,121,129] and protein structures (GEM-PRO) [130,131] and successfully capturing the regulatory effects they have on metabolism. These expansions have greatly increased the scope of questions these models can explore. Constraint based methods, which are powerful tools to estimate the performance of metabolic networks, can predict nonintuitive strategies for metabolic engineering goals such as increasing the yield, productivity or titer of a product of interest. The *in vivo* modeling methods discussed can also be applied to simulate cell free systems.

There have been several mathematical models of cell free protein synthesis; however, the majority of these models have focused exclusively on the transcription and translation processes. These models are mostly systems of ordinary differential equations (ODEs) based upon saturation or Hill-like kinetic expressions. As an early example, Karzbrun and coworkers developed a coarse-grained model of transcription and translation for *E. coli* cell free extract [132]. To simplify calculations, this model was based on four enzymes and ten parameters. Transcription and translation processes were assumed to follow Michaelis-Menten kinetics. The authors noted that the protein synthesis rate began to exponentially decay after 1 hour, so their study focused on the first hour of the cell free experiment. This decay was attributed to resource depletion and waste accumulation. Stögbauer and coworkers developed a model that accounts for resource consumption and degradation and identified the bottleneck of protein synthesis [133]. Variables representing transcription and translation resources were added to the model, but the exact identities and quantities of these resources were beyond the scope of the study. The authors attempted to use Hill functions to better predict saturation effects of mRNA and their protein of interest but found that the optimized Hill coefficients were close to one, resulting in Michaelis-Menten-like approximations. Protein yield was determined to be a function of template DNA concentration. This work also found that NTP depletion was not the reason for protein synthesis rate decay; for the specific extract used, ribosome degradation was to blame for rate decay. More recently, Neiß and coworkers published a comprehensive experimentally validated model that identified limiting factors of cell free protein synthesis [134]. An unusual characteristic of this model is what the authors described as a hybrid black box approach: transcription processes were simplified, while the model for translation was detailed. The entire model was a large system of differential algebraic equations (DAEs); a system of eight algebraic equations and over 400 ODEs. Using sensitivity

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analysis, Neiß found that cell free protein synthesis rates were limited by concentrations of tRNA and elongation factor Tu. A model that captured resource competition in gene networks was published by Gyorgy and Murry [135]. For a two-protein expression system, simulations that considered both products agreed with experimental data. This model was also applied to predict possible product concentrations in multiple-protein expression systems and compare different cell free extracts. The authors concluded that resource competition was a key consideration in the design of synthetic gene circuits. The cell free protein synthesis models discussed thus far have been based on experiments in which DNA serves as the template. RNA genetic circuitry has also been explored in the cell free platform, and mathematical models for the system have been developed. Transcriptional regulating RNAs are of interest because they bypass the need for regulatory proteins [136]. In the context of circuit design, regulatory RNAs can be used to create various logic gates and cascades [137,138]. The first experimentally validated model of a synthetic RNA circuit was published by Hu and coworkers [139]. The model contained 8 ODEs and 13 previously unknown parameters. These parameters were estimated based on results from sensitivity analysis guided experimental design. Taken together, models of transcription, translation, resource competition, and gene regulatory circuits have provided useful information for designing new systems; however, they have each provided an incomplete representation of cell free protein synthesis. CFPS does not just rely on transcription and translation processes, but instead depends upon central carbon metabolism to meet energy and carbon precursor requirements. Thus, more sophisticated models are needed that integrate metabolic pathways with transcription and translation processes. Ultimately, an integrated framework could perhaps provide insights into the limitations of CFPS and provide strategies for improving CFPS performance metrics such as carbon yield, energy efficiency and productivity.

Modeling the integration of cell free transcription and translation processes with metabolic pathways remains in its infancy, with few published mathematical models [140–142]. Horvath and coworkers developed an ensemble of dynamic *E. coli* CFPS models using estimated kinetic parameter sets [142]. This work built upon the hybrid cell free modeling approach of Wayman and colleagues, which integrates kinetic modeling with a rule-based description of allosteric control [143]. Parameter sets were estimated based on measurements of metabolite, amino acid, and protein concentrations from CFPS reactions. By simulating reaction group knockouts, Horvath et al. demonstrated that cell free metabolism and protein synthesis depend mainly on oxidative phosphorylation and glycosis. To circumvent computationally expensive parameter estimation, Vilkhovoy and coworkers [141] developed an experimentally validated constraint-based model of CFPS which integrated the expression of a protein product with the supply of metabolic precursors and energy (Fig. 2). This model coupled transcription and translation processes with available resources using six adjustable parameters. The sequence specific constraint based model of CFPS offered a novel means to *a priori* estimate the performance of a cell free system, using only a limited number of adjustable parameters. Taken together, mathematical modeling of metabolism has proven useful for applications across systems biology, but there is a need for comprehensive metabolic models of cell free reactions. However, as experimental methods are developed for cell free e.g., [144], and benchmark cell free data sets are published, we expect the metabolic modeling community will address this shortcoming.

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5. Conclusion

Cell free systems have become useful and versatile platforms for protein and chemical synthesis, with applications in industries such as pharmaceuticals and energy. With the recent advances in extract preparation, improvements in energy regeneration mechanisms, addition of post-translational modifications, and the ability to perform high throughput reactions in continuous systems such as microfluidic chips, cell free systems have proven to be a valuable bioengineering platform, and a viable alternative to traditional living cells. They have also become a valuable investigative tool for metabolic engineering research given the tunability of reaction conditions and the direct access to metabolites without cell wall interference. However, in order for cell free systems to become a mainstream technology for advanced applications, we must first understand their performance limits and energy costs. A critical tool towards this goal is mathematical and computational modeling which provides systems-level detail on these parameters. Moreover, these models also facilitate the generation of metabolic engineering strategies, for example assisting in making the appropriate genetic manipulation for a desired function, which is not always intuitive. The broad review of literature presented here has highlighted several experimental and computational opportunities that could be addressed in future work. For example, a more detailed description of transcription and translation reactions has been utilized in genome scale metabolic engineering models e.g., O'Brien et al [121]. These template reactions could be adapted to a cell free system, allowing us to consider important facets of protein production, such as the role of chaperones in protein folding. In addition, post-translational modifications such as glycosylation that are important for the production of therapeutic proteins could also be included in the next generation of models. Finally, constraint based modeling could be extended to multi-protein synthetic circuits, RNA circuits or small molecule production. There are also opportunities to explore with regard to the preparation and manipulation of cell extracts. For example, enabling selective enzyme deletions directly in cell free extracts, without having these deletions stem from the extract preparation process, could be a game changing technology. Taken together, continual advancements in the modeling and experimental design have paved the way for cell free systems to become valuable tools for molecular biology research, and a promising platform for manufacturing of valuable biotherapeutics and chemicals, which spans from the micro- to the industrial scale.

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Abbreviations

The following abbreviations are used in this manuscript:

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CFPS	cell free protein synthesis
TXTL	transcription and translation
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
NTP	nucleoside triphosphate
ATP	adenosine triphosphate
GTP	guanosine triphosphate
mRNA	messenger RNA
tRNA	transfer RNA
PURE	Protein synthesis Using Recombinant Elements
ODE	ordinary differential equation
CFME	cell free metabolic engineering
FBA	flux balance analysis
MFA	metabolic flux analysis
ME	metabolic engineering

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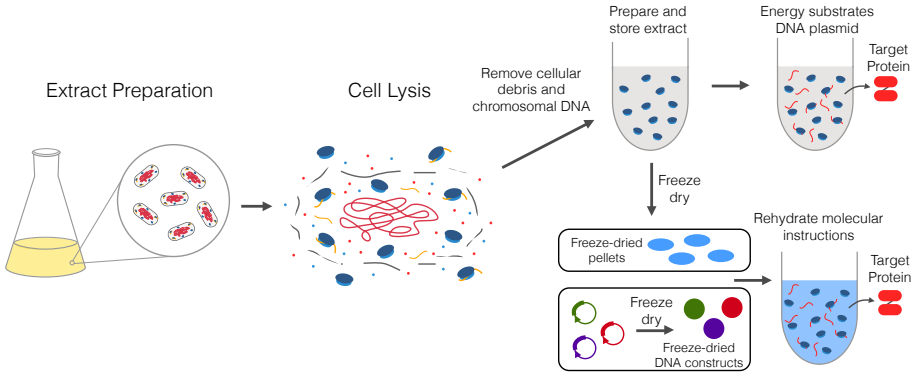

Figure 1. Schematic of cell free protein synthesis. Cell extract is prepared by cell lysis and cellular debris and chromosome DNA is removed. An energy source along with necessary amino acids, nucleotides, and cofactor are added to the cell free reaction. Template DNA of the target protein is added. The target protein is then easily purified from the cell free system. Alternatively, cell free extract can be freeze dried into pellets and paired with lyophilized DNA. Through the simple addition of water, proteins can be manufactured on site and on demand. Figure adapted from [102,145].

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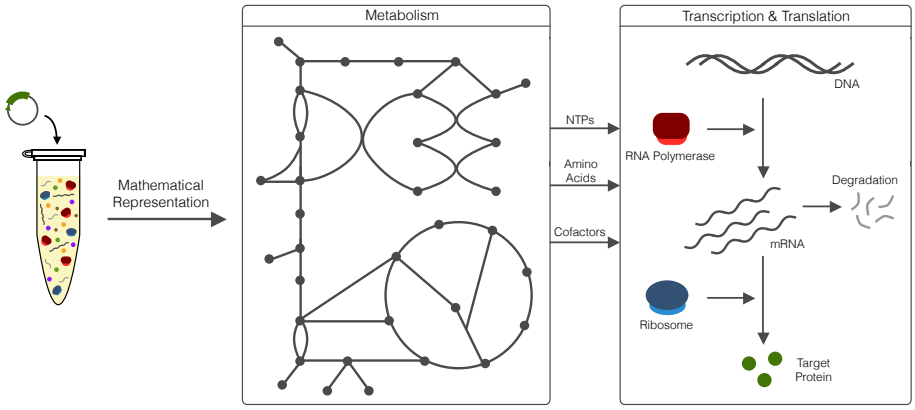

Figure 2. Schematic of the integration of transcription and translation processes integrated with metabolism. Transcription and translation processes demand macromolecular precursors (e.g. NTPS, amino acids and cofactors) from metabolism for gene expression. The target protein in turn can affect enzymatic flux (orange arrow) or the target protein is synthesized as a product (green arrow). The integrated framework is represented as a stoichiometric matrix of metabolites participating in certain reactions, where the flux is estimated subject to constraints, a pseudo-steady state assumption and an objective function. Figure adapted with permission from [141].