ACS SyntheticBiology

Research Article

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Characterizing and Alleviating Substrate Limitations for Improved *in vitro* Ribosome Construction

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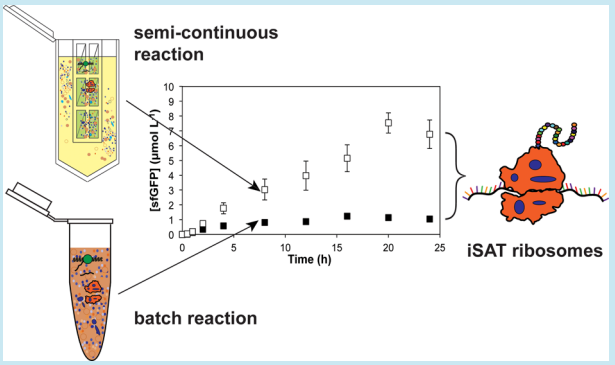
Supporting Information

ABSTRACT: Complete cell-free synthesis of ribosomes could make possible minimal cell projects and the construction of variant ribosomes with new functions. Recently, we reported the development of an integrated synthesis, assembly, and translation (iSAT) method for *in vitro* construction of *Escherichia coli* ribosomes. iSAT allows simultaneous rRNA synthesis, ribosome assembly, and reporter protein expression as a measure of ribosome activity. Here, we explore causes of iSAT reaction termination to improve efficiency and yields. We discovered that phosphoenolpyruvate (PEP), the secondary energy substrate, and nucleoside triphosphates (NTPs) were rapidly degraded during iSAT reactions. In turn, we observed a significant drop in the adenylate energy charge and termination of protein synthesis. Furthermore, we identified that the accumulation of inorganic phosphate is inhibitory to iSAT. Fed-batch replenishment of PEP and magnesium glutamate (to offset the inhibitory effects of accumulating phosphate by repeated additions of PEP) prior to energy depletion prolonged the reaction duration 2-fold and increased superfolder green fluorescent protein (sfGFP) yield by ~75%. By adopting a semi-continuous method, where passive diffusion enables substrate replenishment and byproduct removal, we prolonged iSAT reaction duration 5-fold and increased sfGFP yield 7-fold to 7.5 ± 0.7 μmol L^{-1}. This protein yield is the highest ever reported for iSAT reactions. Our results underscore the critical role energy substrates play in iSAT and highlight the importance of understanding metabolic processes that influence substrate depletion for cell-free synthetic biology.



KEYWORDS: *cell-free synthetic biology, ribosome, Escherichia coli, in vitro transcription and translation, iSAT*

The translation system (the ribosome and associated factors) is the cell's factory for protein synthesis. The extraordinary biosynthetic capacity of the ribosome has driven extensive efforts to harness it for novel functions.[1−3] Previous efforts in ribosome construction *in vitro* have included the elucidation of mechanisms underlying the assembly and action of the translation apparatus,[4−6] the building of minimal cell environments to study the origins of life,[2,7] and the directed evolution of ribosomes to select for enhanced functions or altered chemical properties.[3,8,9] However, the construction of functional ribosomes *in vitro* is a substantial technical challenge. First, the *Escherichia coli* 70S ribosome, at 2.4 MDa, is a large macromolecular complex of many parts. It is composed of a 50S large subunit containing 33 ribosomal proteins (r-proteins), 23S ribosomal RNA (rRNA), and 5S rRNA as well as a 30S small subunit containing 21 r-proteins and 16S rRNA.[10,11] Second, the ribosome assembly process is intricate and highly coordinated. rRNA transcription and ribosome assembly occur simultaneously, proceeding through an alternating series of rRNA conformational changes and protein-binding events.[11−13] Complicating matters, parallel assembly pathways are known to exist.[14] Third, ribosome biogenesis is rapid. To support bacterial log-phase growth with a 40 min doubling time, ribosomes must be produced at a rate of ~8 ribosomes per second to achieve concentrations of 20,000 ribosomes per cell.[15,16]

Despite the complexity of ribosome biogenesis, *in vitro* reconstitution of ribosomes from purified native rRNA and r-proteins has been achieved. Advances stemming from conventional reconstitutions have greatly contributed to our knowledge of ribosome structure and function.[11,14,17−23] However, conventional reconstitution of *E. coli* ribosomes is inefficient, especially for the 50S subunit.[24,25] Additionally, traditional approaches do not mimic the natural coupling of rRNA synthesis and ribosome assembly.[24−27]

To address these challenges, we recently developed an integrated rRNA synthesis, ribosome assembly, and translation technology (termed iSAT) for the construction of synthetic *E. coli* ribosomes in ribosome-free, crude S150 cell lysates (Figure 1A).[28,29] The iSAT method offers several advantages for constructing synthetic ribosomes *in vitro*. First, in contrast to

Received: May 5, 2014
Published: July 31, 2014

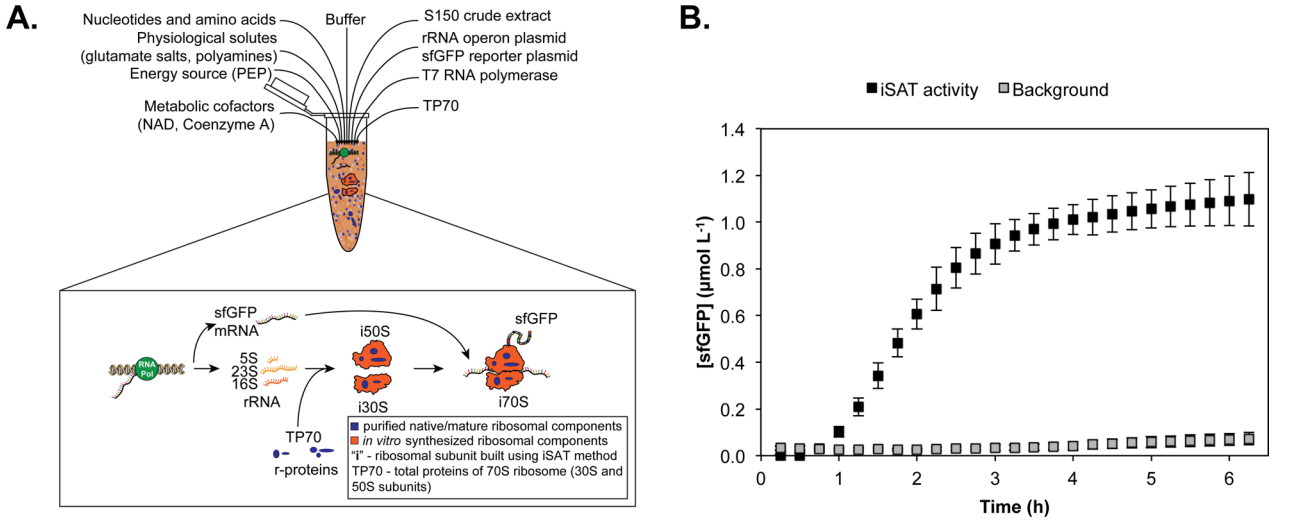


Figure 1. *In vitro* integrated synthesis, assembly, and translation (iSAT) method of constructing ribosomes. (A) Cartoon schematic of iSAT reactions. DNA plasmid encoding rRNAs and reporter protein, substrates (NTPs, amino acids), energy source (PEP), T7 RNA polymerase (RNA Pol), cofactors, physiological salts, purified total proteins of the 70S ribosome (TP70), and S150 ribosome-free extract are mixed and incubated in a microcentrifuge tube at 37 °C. T7 RNA Pol transcribes both reporter mRNA and rRNAs. Synthesized rRNAs assemble with TP70 to form highly active 70S ribosomes (i70S) that then translate reporter protein from mRNA. (B) sfGFP reporter synthesis in iSAT batch reactions over time. Fifteen microliter iSAT batch reactions programmed to synthesize sfGFP were carried out at 37 °C for 6 h. Black boxes denote iSAT activity. Gray boxes denote iSAT reactions without the rRNA operon plasmid as a negative control, which demonstrate background ribosomal activity from the extract alone. Values represent averages and error bars represent standard deviation for at least three independent experiments.

conventional schemes, iSAT mimics co-transcription of rRNA and ribosome assembly as it occurs *in vivo*.[18] Specifically, iSAT ribosomes assemble from *in vitro* transcribed rRNA and purified native ribosomal proteins of *E. coli* 70S ribosomes (TP70), translating reporter protein in the same reaction.[28,29] Second, iSAT has demonstrated improved protein synthesis activity from ribosomes with *in vitro* transcribed 23S rRNA as compared to classical reconstitution methods. In recent work, Fritz and Jewett have shown that iSAT ribosomes now display ∼85−100% protein synthesis activity as compared to that of ribosomes assembled from purified native, post-transcriptionally modified rRNA.[29] This differs from conventional *E. coli* ribosome reconstitution methods, where 50S subunits assembled from *in vitro* transcripts of *E. coli* 23S rRNA have ∼0.02−3% of the activity of those reconstituted from mature rRNA.[24,25,30] Third, in contrast to using fragment reactions where single peptide bonds are formed on isolated 50S subunits, the measure of functional activity in iSAT reactions is the synthesis of full-length, easy to monitor reporter proteins such as superfolder green fluorescent protein (sfGFP) and luciferase.

Despite these advantages for *in vitro* ribosome assembly using iSAT, batch reactions currently last only 4 h before reaction termination.[28,29] Prolonging the duration of translation will enable higher product yields, which is important for technological applications (e.g., constructing ribosomal variants with novel functionalities) and efforts to achieve the break-even milestone of ribosomes that are capable of constructing ribosomes (7434 peptide bonds are needed to make a ribosome equivalent).[28]

Stabilizing reaction substrate concentrations without the concomitant accumulation of harmful side products has underpinned the growth of the cell-free synthetic biology field.[31−34] Focusing on substrate availability instead of protein production has led to major advances in S30 crude extract based cell-free protein synthesis (CFPS) development,[31,33,35−40] enabling bacterial protein synthesis yields exceeding grams of protein per liter reaction and opening the way to clinical manufacturing applications at the 100 L scale.[41,42]

Here, we sought to assess substrate stability in the iSAT system and to develop strategies for prolonging reaction duration to achieve higher product yields. Specifically, we quantified the concentrations of the secondary energy source, phosphoenolpyruvate (PEP), nucleoside tri-, di-, and monophosphates (NXPs), and amino acids in batch iSAT reactions over time using high-performance liquid chromatography (HPLC) analysis. Furthermore, we monitored the accumulation of the inhibitory byproduct, inorganic phosphate, using a chemical assay. We determined that PEP and NTPs were rapidly depleted in batch iSAT reactions. We also observed that accumulating inorganic phosphate decreased iSAT activity. Substrate depletion was overcome by repeated addition of PEP over an 8 h fed-batch reaction. When combined with additional magnesium ions to offset inorganic phosphate accumulation, PEP replenishment extended the reaction to twice its original duration and increased reporter protein yields by 75%. We then adopted a semi-continuous feeding method where substrates and byproducts passively diffuse between the iSAT reaction and a substrate reservoir to sustain small molecule concentrations necessary to keep the reaction active. Here, protein synthesis yields improved and reaction duration lengthened from 1.1 ± 0.1 μmol L^{-1} sfGFP in 6 h batch reactions to up to 7.5 ± 0.7 μmol L^{-1} sfGFP in 24 h semi-continuous reactions. This improvement in the iSAT system makes possible new applications, such as the construction of ribosomal variants at higher yields. Furthermore, it sets the stage for purely *in vitro* ribosome synthesis, the fundamental breakthrough needed for building a synthetic chemical system capable of self-replication and evolution from small molecule building blocks.[2,7,43−48]

dx.doi.org/10.1021/sb5002467 | *ACS Synth. Biol.* 2015, 4, 454−462

RESULTS AND DISCUSSION

Quantitative Substrate Profiles in iSAT Reactions. To begin, we assessed the sfGFP synthesis activity of 15 μL iSAT batch reaction for 6 h at 37 °C. Samples were taken every 15 min to profile sfGFP production. Measurements of sfGFP yield over the course of 6 h iSAT reactions demonstrated an initial lag in sfGFP synthesis during the first 0.5 h and then a linear rate of reporter synthesis for 2 h before protein synthesis rate declined and reactions effectively terminated. In total, 1.1 ± 0.1 μmol L^{-1} sfGFP was synthesized in 6 h batch reactions (Figure 1B). Although no sfGFP fluorescence was detectable until 30 min after the start of iSAT reactions, the delay in reporter synthesis was not attributed to folding. Synthesis of another reporter protein, luciferase, in iSAT showed a similar lag time (Supporting Information Figure 1). Moreover, purified 70S ribosomes in our system do not show such a delay.[29]

To identify the causes of reaction termination, we measured small molecule substrate concentrations in the iSAT system at selected time points in batch reactions using HPLC. Historically, nonproductive energy consumption, secondary energy source depletion, and amino acid exhaustion have been identified as the primary reasons for early termination of cell-free protein synthesis systems that use plasmid DNA templates.[40,49] Thus, we focused our attention on measuring these reaction components.

To gain a sense of the overall energy state of the system, we first measured the secondary energy source, PEP, in iSAT reactions over time (Figure 2A). PEP was depleted rapidly, with less than 0.1% of the initial PEP concentration remaining at 2 h. Not surprisingly, the depletion of PEP, a phosphorylated molecule, was coupled with a rapid increase in inorganic phosphate (Figure 2B). Inorganic phosphate has been shown to accumulate in CFPS systems that use phosphorylated energy substrates and sequester magnesium necessary for operation of the translation apparatus.[31,50] We observed that inorganic phosphate concentrations reach 50 mM 3 h into the reaction (Figure 2B). This sum total (~50 mM) is consistent with the phosphate liberated from PEP and nucleoside triphosphates (NTPs) initially added to the iSAT reaction. To evaluate the effect of phosphate on the system, potassium phosphate at pH 7.0 was added to the iSAT batch reaction in known concentrations (Supporting Information Figure 2). When more than 50 mM additional phosphate was added, sfGFP production was reduced by over 90%, with no production at 100 mM additional phosphate.

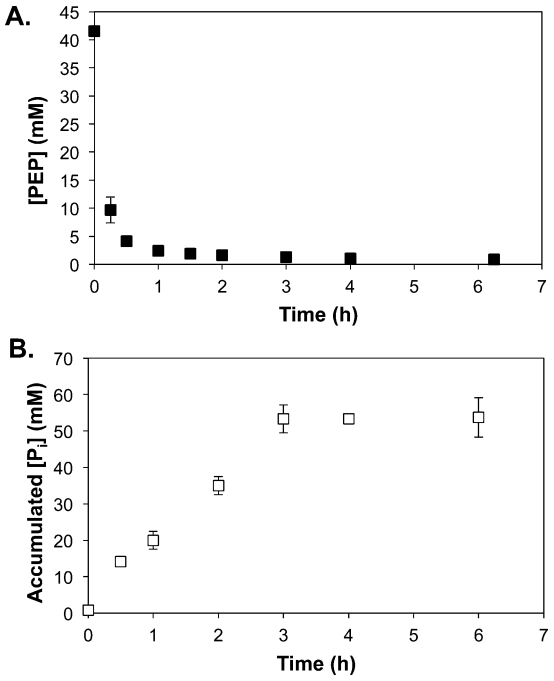


Figure 2. Rapid phosphoenolpyruvate (PEP) depletion in iSAT correlates with inorganic phosphate (P_i) accumulation. PEP (A) and P_i (B) concentrations are shown over the course of batch iSAT reactions. Values represent averages and error bars represent standard deviation for at least three independent experiments.

Once the secondary energy source is depleted, iSAT reactions would, in principle, lose their ability to regenerate NTPs, restricting further system productivity. Thus, we used quantitative HPLC analysis of the nucleotide pool to track each species over time. NTP concentrations, similar to PEP concentrations, decreased rapidly over the first 2 h of the reaction (Figure 3A). This timing corresponded with the exhaustion of the PEP supply. As NTPs were consumed, nucleoside diphosphates (NDPs) and nucleoside monophosphates (NMPs) accumulated sequentially (Figure 3B,C). NDPs did not accumulate over 0.6 mM each and were quickly converted to their monophosphate form, with NMP concentrations stabilizing shortly after 4 h. Notably, PEP and NTP depletion were independent of protein synthesis. For example, negative control iSAT reactions run under standard conditions without the presence of rRNA operon plasmid did not synthesize any measurable sfGFP but still consumed PEP and NTPs (Supporting Information Figure 3). Our observation is consistent with previous reports of nonproductive substrate consumption in crude S30 cell lysate protein synthesis systems, which have demonstrated that enzymes in the lysate can deplete substrates in the absence of protein synthesis.[31,40,49] Notably, substrate depletion in iSAT reactions was faster than that in S30 crude extract-based CFPS.[36]

Evaluating the adenylate energy charge (E.C.), defined by Atkinson as the overall status of energy availability in the system (Eq 1), allows us to explore when energy in the system becomes limiting.[51]

$$\text{E.C.} = \frac{[\text{ATP}] + \frac{1}{2}[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]} \quad (1)$$

It has been reported that *in vivo* E.C. measurements below 0.8 indicate energy limitation.[52] In iSAT reactions, this threshold is reached ~1−2 h into the reaction, suggesting that maximal protein synthesis rates cannot be maintained for extended durations (Figure 3D).

In addition to secondary energy source depletion, previous work has demonstrated that consumption of amino acids was a factor leading to the halt of protein synthesis in *E. coli* CFPS from S30 crude extracts.[32,40,53] Therefore, we determined the concentrations of 19 amino acids in the iSAT system during the course of 6 h batch reactions (Supporting Information Figure 4). Seven of the amino acids (histidine, valine, tryptophan, isoleucine, leucine, lysine, and proline) maintained relatively constant concentrations around their initial concentration of 2 mM, whereas the other amino acid concentrations changed significantly. Only aspartic acid was exhausted after 4 h, following sfGFP synthesis termination. Asparagine, glutamine, and serine were depleted but were maintained at nonzero

dx.doi.org/10.1021/sb5002467 | ACS Synth. Biol. 2015, 4, 454−462

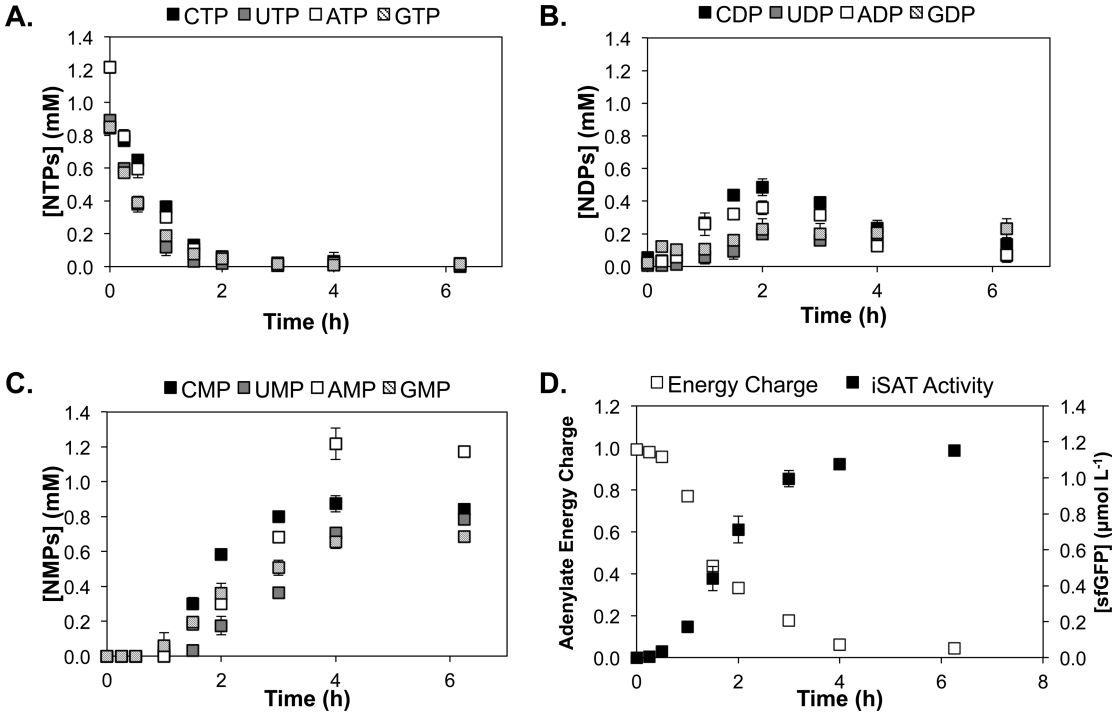


Figure 3. iSAT NXP profile demonstrates energy limitation. Nucleoside triphosphate (NTP) (A), diphosphate (NDP) (B), and monophosphate (NMP) (C) concentrations are shown over the course of batch iSAT reactions. NMPs accumulate as the reaction progresses. (D) Adenylate energy charge, a measurement of the available energy in the reaction, compared to iSAT activity over time. We observed a decrease in synthesis of sfGFP reporter (filled) when adenylate energy charge (open) is depleted. Values represent averages and error bars represent standard deviation for at least three independent experiments.

levels. Alanine and phenylalanine increased significantly, to 8 and 4 mM, respectively. Glutamate was excluded from our analysis because it is in excess in iSAT reactions (≥150 mM). These data suggest that iSAT reactions are not constrained by amino acid depletion, but may be so if energy could be stabilized. Subsequent substrate replenishment studies later confirmed that amino acids were not limiting (see below).

Extending the Duration of iSAT Reactions with Substrate Replenishment. Quantitative characterization of small molecule substrate availability showed that energy limitations restricted the productivity of the batch iSAT system. Thus, we performed fed-batch experiments to determine if the duration of protein synthesis could be extended by replenishing the secondary energy substrate PEP. Since PEP consumption produces inhibitory phosphate in iSAT reactions, we additionally fed magnesium glutamate to offset the deleterious effects of magnesium sequestration.[31] PEP was fed at its initial concentration of 40 mM and the concentration of magnesium glutamate was optimized at 14 mM for highest protein yields (Supporting Information Figure 5). The time of feeding was also optimized for protein yields, with substrate replenishment at 2 h generating the best result (Supporting Information Figure 6). This corresponded with the time of NTP depletion (Figure 3A). Addition of 40 mM PEP and 14 mM magnesium glutamate at 2 h increased sfGFP expression yields to 75% above that of the control without substrate feeding (water supplementation only) over 8 h batch reactions (Figure 4). Repeated addition of PEP and magnesium glutamate at 3.75 h further increased yields (Figure 4), but not substantially. More additions (i.e., more than 2 additions) did not improve protein synthesis.

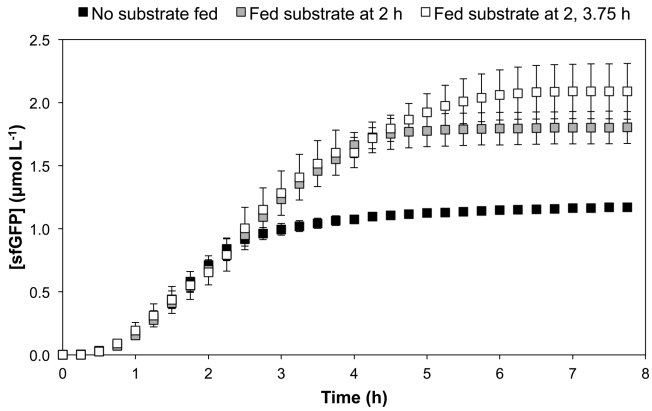


Figure 4. Replenishing PEP and magnesium glutamate during iSAT reaction prolongs protein synthesis and increases overall sfGFP yields. Batch reactions were fed 40 mM PEP and 14 mM magnesium glutamate at 2 h (gray) or at 2 and 3.75 h (white). Batch control reactions (black) were fed equivalent volumes of water. Values represent averages and error bars represent standard deviation for at least three independent experiments.

In subsequent experiments, we showed that supplementation of NTPs or amino acids along with PEP and magnesium glutamate did not have an effect on fed-batch protein yield (Supporting Information Figure 7). Beyond these small molecule substrates, we also investigated the addition of sfGFP plasmid DNA template and T7 RNA polymerase along with PEP and magnesium glutamate because T7 RNA polymerase could be degraded and limit mRNA production.[39] Adding more sfGFP reporter plasmid and T7 RNA polymerase did not improve fed-batch yields (Supporting Information

dx.doi.org/10.1021/sb5002467 | ACS Synth. Biol. 2015, 4, 454−462

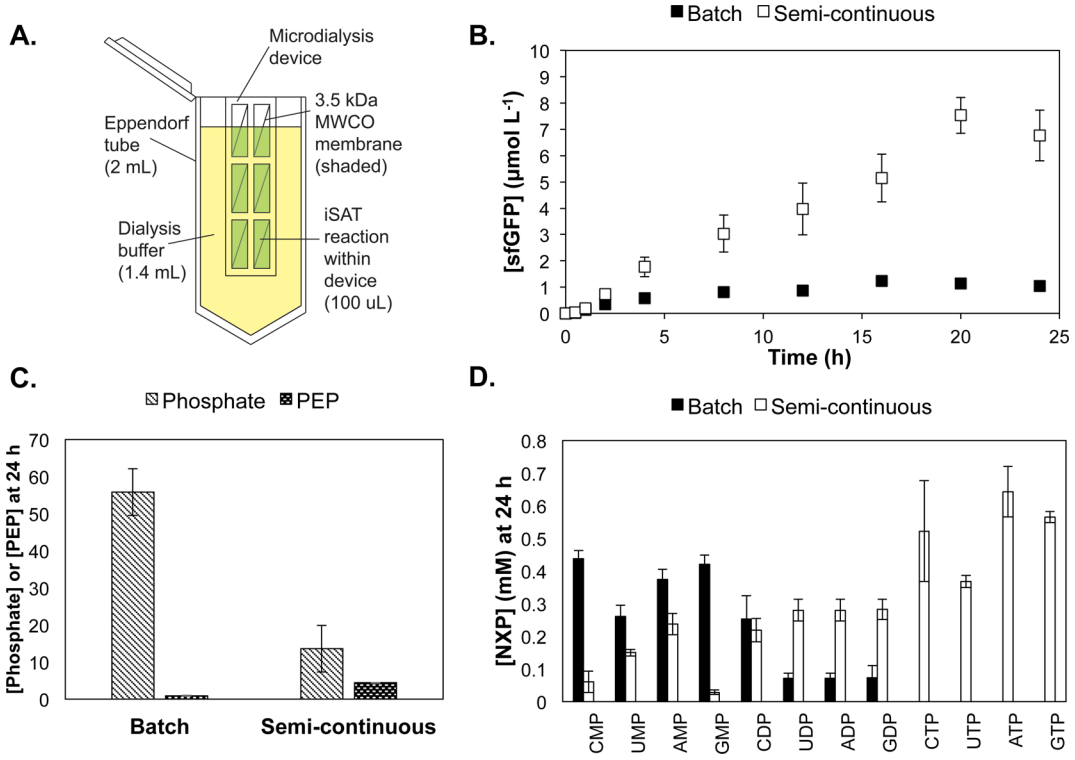


Figure 5. Semi-continuous reaction format prolongs protein synthesis, increases sfGFP yield, and alleviates substrate limitations and phosphate accumulation. (A) Diagram of a semi-continuous setup for running iSAT reactions. iSAT reactions (100 μL) were performed in a microdialysis device with a 3.5 kDa MWCO membrane that interfaced with a 1.4 mL (14× volume) dialysis reservoir containing equimolar concentrations of energy source substrates, cofactors, and salts in a 2 mL microcentrifuge tube. As the reaction progresses, depleted substrates in the reaction are replenished by the dialysis reservoir by passive diffusion, and accumulated byproducts in the reaction diffuse out into the reservoir. (B) Semi-continuous iSAT setup prolongs protein synthesis and improves sfGFP yield. 100 μL iSAT reactions (white) were run at 37 °C in a semi-continuous setup. As a control, 100 μL iSAT batch reactions (black) were run in parallel. Batch reactions terminated by ~6 h with a total sfGFP yield of 1.1 ± 0.1 μmol L^{-1}, whereas semi-continuous reactions terminated by ~20 h with total sfGFP yield of 7.5 ± 0.7 μmol L^{-1}. (C) Semi-continuous iSAT reactions contain higher PEP concentrations and lower phosphate concentrations than batch reactions after 24 h. Concentrations of PEP (striped) and P_i (dotted) in semi-continuous and batch iSAT reactions at reaction termination (24 h). (D) NXP concentrations at semi-continuous and batch iSAT reactions after 24 h. Batch reactions (black) did not have any measurable concentrations of NTPs after 24 h, whereas semi-continuous reactions (white) still contained measurable concentrations of all NTPs. Values represent averages and error bars represent standard deviation for at least three independent experiments.

Figure 7), suggesting that mRNA did not limit iSAT reactions. These data were consistent with RNA gels that showed mRNA was present in batch iSAT reactions after reaction termination (data not shown).

Our quantitative small molecule characterization studies and fed-batch results indicated that secondary energy substrate depletion was most likely the main factor limiting batch iSAT reactions, but that inorganic phosphate ultimately poisoned fed-batch reactions. To test this hypothesis and to improve iSAT protein synthesis, we utilized a semi-continuous method, where substrate replenishment and inhibitory byproduct removal occur simultaneously (Figure 5A). Here, a large volume of reaction reagents is separated from the reaction mixture by a dialysis membrane. Kim and Choi first demonstrated the effectiveness of this reactor design, producing 1.2 mg protein mL^{-1} reaction in S30 crude extract based CFPS.[54] In our setup, iSAT reactions were carried out on the 100 μL scale against a 1.4 mL dialysate buffer, which is composed of substrates and salts at reaction concentrations and is separated from the reaction by a 3.5 kDa MWCO membrane (Figure 5A). Template DNA, RNA, ribosomes, and other proteins from the extract are unable to diffuse across the membrane of this MWCO and thus remain in the reaction chamber. While the control batch reactions were performed at 100 μL reaction volumes to match semi-continuous reactions, the increase in reaction volume relative to our earlier experiments had little effect on sfGFP yield (Supporting Information Figure 8).

We compared 100 μL iSAT semi-continuous and batch yields of sfGFP in 24 h reactions. Semi-continuous iSAT reactions demonstrated a linear rate of sfGFP synthesis of 0.4 μmol L^{-1} h^{-1} for up to 20 h at peak production, compared to only 2 h in batch reactions. The extended protein synthesis duration achieved a maximum total yield of 7.5 ± 0.7 μmol L^{-1} sfGFP after 24 h, a 7-fold improvement over batch methods (Figure 5B). PEP and NTP measurements showed that semi-continuous reactions still contained 4.4 mM PEP and >0.4 mM NTP at 24 h (Figure 5C,D), compared to depletion by only 2 h for batch reactions (Figures 2 and 3). Finally, we observed that inorganic phosphate concentration at the end of semi-continuous reactions was 14 mM, compared to >50 mM at the end of batch reactions (Figure 5C). We note that the volumetric productivity (micromoles of sfGFP per liter of total reagents, including the feeding support solution) is lower than the fed-batch experiments: 0.5 ± 0.1 μmol sfGFP per L total reagents in semi-continuous reactions versus 1.9 ± 0.2 μmol sfGFP per L total reagents in fed-batch reactions. Although the semi-continuous method consumes expensive reagents, our results demonstrated that the exchange of small molecules,

dx.doi.org/10.1021/sb5002467 | ACS Synth. Biol. 2015, 4, 454−462

including limiting substrates and toxic byproducts, prolonged protein synthesis, leading to a higher protein yield. Looking forward, implementing nonphosphorylated energy substrates could avoid phosphate accumulation and address the need for byproduct removal.[34,40]

CFPS platforms are known to experience limited batch synthesis yields, in part due to constraints arising from substrate depletion and inhibitory byproduct accumulation. This problem has been largely overcome in S30 crude extract-based *E. coli* CFPS platforms by activating endogenous energy regeneration pathways and knocking out enzymatic reactions that lead to amino acid consumption.[33,34,36,55] Here, for our S150 crude extract-based iSAT reactions, we identified the primary reason for reaction termination by characterizing substrate profiles in the iSAT system using a systematic approach. We found that energy limitations (specifically loss of PEP availability, NTP depletion, and E.C. drop), as well as inorganic phosphate accumulation, limited the protein synthesis of iSAT reactions. These results in the iSAT system are similar to previous work in *E. coli*- and yeast-based CFPS systems.[31,49,56]

Our careful analysis revealed that despite efforts to replicate essential features of the cytoplasm, such as combined ribosome synthesis and assembly, substrate limitations and inhibitory byproduct accumulation in iSAT reactions are an issue. Our work shows that substrate depletion can be directly addressed by replenishment via fed-batch reactions. Furthermore, we show that semi-continuous reactions alleviate substrate limitations and reduce the deleterious effects of inhibitory phosphate accumulation. For example, at 24 h, PEP, NTPs, and amino acids (Supporting Information Figure 9) are still present in the semi-continuous reaction chamber and inorganic phosphate concentrations are lower than those in batch controls. This indicates that the cause of reaction termination is no longer substrate dependent. We hypothesize that the cause now may be dependent on the half-life of enzyme complexes, such as iSAT-assembled ribosomes, used to carry out protein synthesis as reactions run for 20 h at 37 °C. Future work in this area will focus on characterizing the activity of ribosomes constructed in iSAT reactions.

Our results emphasize the importance of understanding active metabolic reactions in iSAT reactions and, more broadly, *in vitro* synthetic biology. Indeed, by removing substrate limitations and avoiding phosphate inhibition, we were able to synthesize up to 7.5 ± 0.7 μmol L^{-1} sfGFP after 24 h from iSAT-assembled ribosomes containing *in vitro* synthesized rRNA. This is important because it is the highest reported yield of protein from *in vitro* constructed ribosomes, paving the way for purely *in vitro* ribosome synthesis. That goal, the complete synthesis of a cell-free ribosome, is still a considerable challenge because of the "chicken or egg" paradox: we need pre-existing ribosomes that co-synthesize 54 ribosomal (r-) proteins and then we need those r-proteins to be assembled into synthetic ribosomes.[2,7] Beyond enabling ribosomes that make ribosomes, there are still exciting opportunities to improve iSAT reactions. First, since the parent S150 extract source strain has not been previously optimized for iSAT reactions, we expect future efforts will be to design and engineer genomes that lead to improved S150 extract performance and increased synthesis yields. For example, multiplex automated genome engineering (MAGE)[57] could be used to make numerous genomic changes to up- and downregulate enzymes that may effect iSAT productivity. Nonessential metabolic pathways, such as alanine synthesis (Supporting Information Figure 4), could be knocked out if they prove to be detrimental to the overall reaction efficiency. There may also be utility in upregulating chaperones and assembly factors known to support ribosome biogenesis. Over 20 *E. coli* proteins have been identified as ribosomal assembly cofactors, including helicases, GTPases, and chaperones, that facilitate RNA folding and RNA−protein interactions in the formation of the 30S and 50S subunits.[11,58] Not only will studying chaperones be potentially useful from a technological perspective, but also understanding the roles of assembly cofactors, from individual mechanisms to network interactions, could provide fundamental insights into ribosome biogenesis. Beyond genome engineering, there is a need to carefully examine the differences between *in vivo* assembled ribosomes and those made in the iSAT system. Ribosome biogenesis follows multiple parallel paths toward formation of the 70S complex depending on reaction conditions of *in vivo* or *in vitro* reconstitution experiments.[12,14] The kinetic differences between iSAT and *in vivo* ribosome assembly can be used as a tool for understanding the stages of ribosome biogenesis on a slower time scale as well as studying changes in ribosome assembly under different physiological conditions. Finally, quantifying the active number of ribosomes per reaction versus the total number of ribosomes and rRNA transcribed could be crucial for efforts to further improve activity.

In summary, we carried out a systematic study to characterize substrate and byproduct small molecules in the iSAT system. By evaluating substrate concentrations, we determined that the source of early reaction termination resulted from a cascade of events resulting from the loss of the secondary energy substrate, PEP. We went on to show that a semi-continuous reaction format could alleviate these limitations to realize a 7-fold increase in active protein synthesis and the information gained can now be leveraged to improve batch reaction performance. Looking forward, we anticipate that iSAT will contribute meaningfully toward efforts in cell-free synthetic biology to construct and use synthetic ribosomes.

METHODS

S150 Extract Preparation. S150 crude cell-free extract was prepared from MRE600 *E. coli* cells as previously reported.[29] Briefly, MRE600 *E. coli* cells were grown to OD_{600} = 3.0 in 10 L fermentation. Cells were lysed using high-pressure homogenization (~20 000 psig), and the lysate was passed through a sucrose gradient cushion to pellet ribosomes from the lysate, which are then used for TP70 preparation (described below). The supernatant was dialyzed overnight in high salt buffer to remove sucrose and concentrated 6−8-fold to make S150 extract.

Total Protein of 70S Ribosomes (TP70) Preparation. TP70, which stands for total protein of 70S ribosomes, is composed of the purified protein components of ribosomes isolated from *E. coli* crude cell extract in the process of making S150 ribosome-free extract. TP70 was prepared as previously described.[29] Succinctly, the ribosome pellet was resuspended, and its rRNA was removed by acetic acid precipitation. Ribosomal proteins were isolated via acetone precipitation, dialyzed against a urea buffer to unfold, and then dialyzed against a high salt buffer without urea to refold and make TP70.

iSAT Batch Reactions. Integrated synthesis, assembly, and translation (iSAT) reactions (15 μL) were carried out at 37 °C as previously reported,[29] with concentrations of magnesium glutamate, reporter plasmid, and TP70 optimized for maximum

dx.doi.org/10.1021/sb5002467 | *ACS Synth. Biol.* 2015, 4, 454−462

productivity and minimal consumption of parts. For example, we observed that iSAT activity was not statistically different with plasmid levels between 0.1 and 1 mM (Supporting Information Figure 10). Added to the reaction mixture were 12 mM magnesium glutamate, 150 mM potassium glutamate, 1.2 mM adenosine triphosphate (ATP), 0.85 mM guanosine triphosphate (GTP), 0.85 mM cytidine triphosphate (CTP), 0.85 mM uridine triphosphate (UTP), 34 μg mL^{-1} folinic acid, 170.6 μg mL^{-1} *E. coli* tRNA mixture, 2 mM each of 20 amino acids, 0.33 mM nicotinamide adenine dinucleotide (NAD), 0.27 mM coenzyme A (CoA), 57 mM HEPES-KOH, pH 7.6, 4 mM oxalic acid, 1.75 mM spermidine, 1.5 mM putrescine, 40 mM phosphoenolpyruvate (PEP), 0.1 nM reporter plasmid (pY71-sfGFP), 4 nM plasmid pT7rrnb containing 23S ribosomal rRNA, 16S rRNA, and 5S rRNA on a T7 operon, 0.3 mg mL^{-1} T7 RNA polymerase, 0.1 μM total ribosomal protein mixture (TP70), and 0.4 volumes of S150 extract.

iSAT Fed-Batch Reactions. iSAT batch reactions (15 μL) were performed as described above. Then, at given time points, an equivalent volume of feed substrates and/or water were pipetted directly into the reactions to replenish the desired substrate at concentrations specified in the text. Reactions were performed at 37 °C for 8 h.

iSAT Semi-continuous Reactions. 100 μL iSAT reactions were carried out in Pierce 3.5K MWCO Microdialysis Plates (Thermo Fisher Scientific, Rockford, IL). The plates contain microdialysis devices that interface with a dialysis buffer (14× reaction volume), which allow small molecules to passively diffuse between the reaction and the buffer. The dialysis buffer contained 4.7 mM Tris acetate (pH 7.5 at 4 °C), 4.7 mM magnesium acetate, 9.3 mM ammonium acetate, 14 mM potassium acetate, 243 mM potassium glutamate, 1.97 mM spermidine, 1.47 mM putrescine, 0.47 mM dithiothreitol (DTT), 0.23 mM ethylenediaminetetraacetic acid (EDTA), 1.2 mM adenosine triphosphate (ATP), 0.85 mM guanosine triphosphate (GTP), 0.85 mM cytidine triphosphate (CTP), 0.85 mM uridine triphosphate (UTP), 34 μg mL^{-1} folinic acid, 170.6 μg mL^{-1} *E. coli* tRNA mixture, 2 mM each of 20 amino acids, 0.33 mM nicotinamide adenine dinucleotide (NAD), 0.27 mM coenzyme A (CoA), 57 mM HEPES-KOH, pH 7.6, 4 mM oxalic acid, and 40 mM phosphoenolpyruvate (PEP). Reactions were performed at 37 °C for 24 h, noting that we used 1 nM plasmid in the reactions shown in Figure 5. We found that iSAT protein expression yields were higher when using this concentration in iSAT reactions as compared to the 0.1 nM used in the batch reactions described earlier (data not shown), perhaps because template stability was an issue at longer reaction times.

sfGFP Quantification. We used sfGFP as a reporter protein to measure protein synthesis activity of iSAT-constructed ribosomes because its activity can be easily assayed in cell-free reactions by measuring fluorescence. Furthermore, sfGFP synthesized in iSAT reactions is highly active, as reported previously.[29] sfGFP quantification was performed as previously reported.[29,49] sfGFP fluorescence in iSAT batch and fed-batch time courses was measured in a CFX96 real-time thermal cycler (Bio-Rad, Hercules, CA). Samples of iSAT semi-continuous reaction time courses and end-point batch reactions were incubated in sfGFP assay buffer for 15 min at room temperature and measured using BioTek Synergy 2 plate reader (Winooski, VT). Relative fluorescence units from the thermal cycler and plate reader were converted to molar concentrations using a linear standard curve made in house by expressing ^{14}C-leucine-labeled sfGFP in an *E. coli* PANOx CFPS reaction and relating RFUs to trichloracetic acid precipitable soluble protein yield.

Inorganic Phosphate Quantitative Assay. Inorganic phosphate was measured as previously reported.[49] Briefly, quantitative analysis of inorganic phosphate was performed using the EnzChek Phosphate Assay Kit (Life Technologies, Carlsbad, CA). iSAT samples were frozen on liquid nitrogen to quench the reaction and later thawed and assayed. To measure phosphate concentration, samples were diluted 500-fold with nanopure water and compared to a linear phosphate standard ranging from 2 to 150 μM. Absorbance was read at 360 nm following the manufacture's instructions.

Nucleotide, Amino Acid, and Phosphoenolpyruvate Concentration Analysis. High-performance liquid chromatography (HPLC) analysis was used to measure nucleotides, amino acids, and PEP as previously reported.[49] Phosphoenolpyruvate (PEP) was also measured using the previously described nucleotide analysis method, with detection at 210 nm. All metabolite concentrations were determined by comparison to a standard calibration.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

M.C.J., Y.L., and B.R.F. conceived the study. Y.L., M.J.A., and J.A.S. designed experiments. Y.L. and M.J.A. performed experiments and analysis. Y.L. and M.C.J. wrote the manuscript. B.R.F., M.J.A., and J.A.S. edited the manuscript. M.C.J. provided a supervisory role.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the Office of Naval Research (N00014-11-1-0363).

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