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Graphical Abstract (will be published online)

Research Article

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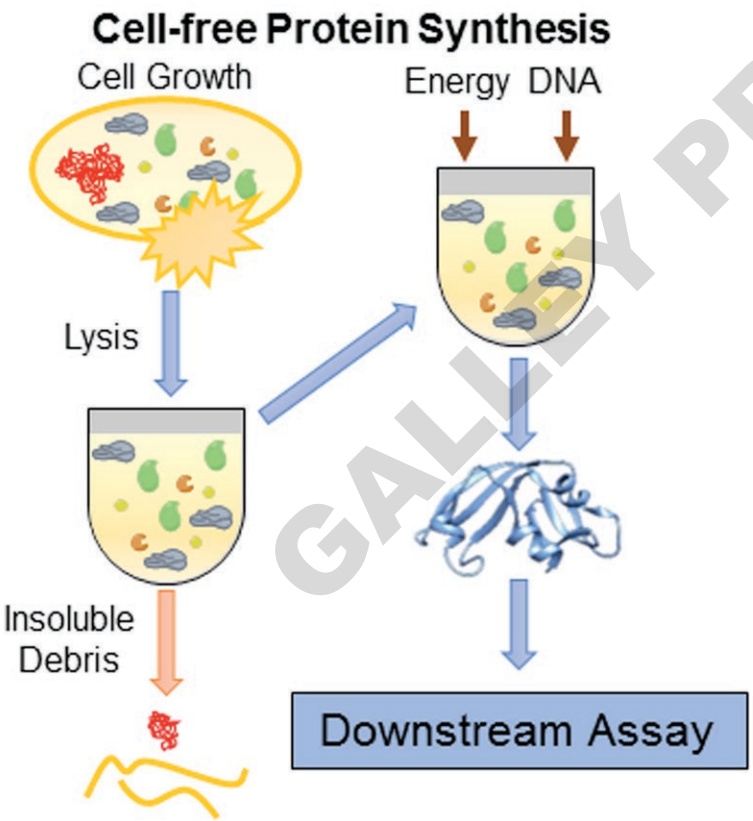

Cytotoxic biotherapeutics, such as the protein onconase, are promising anticancer alternatives to traditional chemotherapies. Such proteins pose challenges to recombinant in vivo expression like cytotoxicity and aggregation. Cell-free protein synthesis overcomes these challenges. In this study, cell-free expressed onconase is soluble and active, allowing for immediate downstream characterization without the need of purification. The authors further demonstrate the robustness of lyophilized cell-free systems for such applications and report that these systems remain active up to one year stored above freezing.

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Cell-free protein synthesis of a cytotoxic cancer therapeutic: Onconase production and a just-add-water cell-free system

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Biotherapeutics have many promising applications, such as anti-cancer treatments, immune suppression, and vaccines. However, due to their biological nature, some biotherapeutics can be challenging to rapidly express and screen for activity through traditional recombinant methods. For example, difficult-to-express proteins may be cytotoxic or form inclusion bodies during expression, increasing the time, labor, and difficulty of purification and downstream characterization. One potential pathway to simplify the expression and screening of such therapeutics is to utilize cell-free protein synthesis. Cell-free systems offer a compelling alternative to in vivo production, due to their open and malleable reaction environments. In this work, we demonstrate the use of cell-free systems for the expression and direct screening of the difficult-to-express cytotoxic protein onconase. Using cell-free systems, onconase can be rapidly expressed in soluble, active form. Furthermore, the open nature of the reaction environment allows for direct and immediate downstream characterization without the need of purification. Also, we report the ability of a "just-add-water" lyophilized cell-fee system to produce onconase. This lyophilized system remains viable after being stored above freezing for up to one year. The beneficial features of these cell-free systems make them compelling candidates for future biotherapeutic screening and production.

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1 Introduction

Biopharmaceuticals is a burgeoning 140 billion USD industry and continues to grow [1]. The industry has the potential to improve and save lives by tapping into the vast evolved diversity and function that nature has to offer. This is epitomized by the success of biological therapeutics, as seven of the top ten grossing prescription drugs are recombinant antibodies or fusion proteins [2]. Production and screening systems for recombinant antibodies are considerably streamlined and continually improving [3]. However, as new non-antibody biotherapeutics are investigated, production and screening technologies will need to adapt accordingly.

Cancer is the second leading cause of death in the United States, presenting a compelling target for current and future biologics (CDC, *Deaths: Final Data for 2013*) [4]. Non-prophylactic anticancer treatments are by definition cytotoxic. Cytotoxic anticancer biologics may prove difficult to overexpress, scale-up, or screen due to their intrinsically toxic properties. For example, onconase (ONC) is a known ribonuclease that is in clinical trials for treatment of malignant mesothelioma and has demonstrated potency against multiple other forms of cancer [5–7]. However, ONC is derived from the oocytes of the Northern Leopard

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Abbreviations: CFPS, cell-free protein synthesis; **GFP**, green fluorescent protein; **IC_{50}**, inhibitory concentration of 50% activity; **IPTG**, isopropyl-1-thio-b-D-glactopyranoside; **IRV**, initial reaction volume; **lyo-CFPS**, lyophilized CFPS; **MbRS**, *Methanosarcina barkeri* aminoacyl-tRNA synthetase; **MTT**, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide; **ONC**, onconase; **ONC_{CFPS}**, ONC produced in CFPS; **ONC_{invivo}**, ONC produced in vivo

* These authors contributed equally to this work.

Frog, which are time and labor intensive to harvest and culture [8, 9]. Furthermore, downstream evaluation and screening of ONC requires purification including anion exchange, cation exchange, and finally size-exclusion chromatography [5].

Attempts at alternative recombinant expression methods for ONC have had limited success. Producing ONC in the most widely used recombinant expression system, *Escherichia coli*, results in the formation of inclusion bodies, requiring laborious purification and refolding procedures taking three or more days [10]. Although strides have been made to recombinantly express and secrete ONC in yeasts, these systems also require laborious purification and concentration steps prior to downstream evaluation and screening [11].

In efforts to circumvent the production limitations of cell-based systems and enable the rapid contiguous evaluation of the protein, we propose cell-free protein synthesis (CFPS) of ONC. Cell-free systems feature open reaction environments that can be readily manipulated and monitored for optimal reaction conditions [12, 13]. Furthermore, CFPS systems are generally less sensitive to cytotoxic elements, as there are no cells to keep alive [14]. Recently, the lyophilization of CFPS reagents has enabled long-term storage at non-ideal temperatures (up to 90 days above freezing), creating a robust "just-add-water" expression system [15]. These traits make CFPS a compelling alternative for the production and screening of difficult-to-express biomolecules, such as cytotoxic, insoluble, and membrane proteins [16, 17].

E. coli-based cell-free systems are particularly robust recombinant expression platforms with reported yields as high as 2.3 mg/mL reaction [18]. These systems have been utilized to produce diverse products, such as cytotoxic viral proteins, oxygen-sensitive Fe-Fe enzymes, and therapeutic peptides [14, 19, 20]. Other applications of *E. coli*-based CFPS have significantly improved protein solubility and avoided the difficulties associated with inclusion body formation [21, 22].

Here we demonstrate the robust production of active ONC in *E. coli*-based CFPS. The cell-free produced ONC (ONC_{CFPS}) is highly soluble during and after overexpression. ONC_{CFPS} exhibits similar protein synthesis inhibition and improved anticancer activities compared to in vivo produced ONC (ONC_{invivo}). Finally, we show how ONC_{CFPS} could be produced on-demand by stable and portable lyophilized CFPS systems. We also report the ability of "just-add-water" lyophilized cell-free systems to be active for up to one year when stored above -80°C. In short, cell-free protein synthesisis a robust alternative to produce and screen the difficult-to-express protein ONC. The ability to rapidly produce soluble, active proteins has implications in engineering, screening, and producing current and future biotherapeutics. Such technologies will be essential as medicine becomes more advanced, personal and on-demand.

2 Materials and methods

2.1 Cell growth and extract preparation

Cell extracts were prepared as previously described with the following specifications [22]. *Escherichia coli* BL21-Star™ (DE3) (Invitrogen, Carlsbad, CA) was grown serially from glycerol stocks at 37°C and 280 rpm as follows: 5 mL LB media overnight, 100 mL LB media until OD_{600} of 2.0, and 1 L 2YT media. Growths were induced by addition of 1 mL 1 M isopropyl-1-thio-β-D-galactopyranoside (IPTG) at OD_{600} 0.4–0.7 and monitored until the end of log-phase growth. Cells were harvested by centrifugation at 6000 ×*g*, 4°C for 15 min and subsequently washed with buffer A (10 mM Tris-acetate, 14 mM magnesium acetate, 60 mM potassium glutamate, 1 mM dithiothreitol, pH 8.2). Cells were again centrifuged as before, resuspended in 1 mL buffer A per gram wet cells, and lysed as previously described [22]. Lysates were centrifuged for 30 min at 16 000 ×*g*, 4°C. The supernatant was removed, aliquotted, flash frozen with liquid nitrogen, and stored at –80°C until use. Lyophilized extracts were prepared as previously described [15].

2.2 In vivo expression of onconase

Recombinant onconase gene (NCBI Accession# 1PU3_A) was codon optimized and custom synthesized (DNA 2.0, Menlo Park, CA) for improved expression in *E. coli*. The optimized gene was cloned into the T7-promoted expression plasmid pJ411-KanR with a Q2E mutation and C-terminal 6×His tag, resulting in plasmid pJ411-Onc.

E. coli BL21-Star™ (DE3) was transformed with pJ411-Onc. Cells were grown serially as described above with the following specifications. The final stage of cell growth was 1 L Terrific Broth media. Growths were induced at OD_{600} 0.4–0.7 by addition of 1 mL 1 M IPTG and grown overnight. Cells were harvested, washed, and lysed as above. ONC_{invivo} was purified from inclusion bodies in the cell lysate pellet and refolded as previously described [23]. To verify the molecular mass, ONC_{invivo} was desalted and analyzed using an Agilent Technologies 6230 TOF LC/MS (Agilent, Palo Alto, CA) (Supporting information Fig. S1).

2.3 Cell-free protein synthesis of onconase

Cell-free protein synthesis (CFPS) ONC production was performed using the PANOxSP system, as previously described [15]. Reactions were performed for 3 h 15 min at 37°C. Production yields were calculated by incorporation of radiolabeled C^{14}-Leucine, as previously described [24]. Yields from CFPS reactions diluted during the reaction incubation were calculated based on the initial reaction volume (IRV). CFPS reactions for protein synthesis inhibition assays were performed without dilution. Reac-

tion volumes were scaled between 50 µL and 1 mL with no observable change in yield (Supporting information, Fig. S2).

2.4 Anticancer activity assay

To assay the anticancer properties of ONC, we measured the cell viability of breast cancer cells (MCF7) using an MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide) assay [25]. MCF7 cells were grown in Dublecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Once confluent, cells were trypsinized (trypsin-EDTA 0.05%) and plated into a clear culture-grade 96-well plate at 3000 cells per well and grown for 24 h in 100 µL fresh media. Before treatment, the growth media were replaced with 100 µL fresh media, treatment sample, and up to 50 µL PBS (pH 8.0) for a total well volume of 150 µL. After 48 h incubation, treatment solution was aspirated, wells were washed with 200 µL PBS, and finally filled with 90 µL fresh media and 10 µL of 5 mg/mL MTT in PBS. After 4 h incubation, MTT solution was aspirated and replaced with 75 µL DMSO. The plate was incubated for 15 min at room temperature and absorbance was measured at 540 nm with background absorbance of 720 nm subtracted.

2.5 tRNA purification

Total RNA was purified from BL21-Star™ (DE3) extract using two rounds of acid phenol:chloroform:isoamylalcohol (Ph:Chl:IA) 125:24:1 extraction as follows. Ph:Chl:IA was added to one volume of extract, vigorously vortexed, and centrifuged at >16 000 × *g*, 4°C for 30 min. After centrifugation and transferring the aqueous phase to a new tube, a mix of sample:isopropyl alcohol:8 M ammonium acetate 100:145:45 by volume was used to precipitate total RNA, and the pellet was washed with cold 95% and 70% ethanol. The alcohol precipitation and wash was repeated two times. The resulting pellet was dissolved in a mixture of 0.73 M LiCl, 10 mM sodium acetate pH 4.5 to precipitate large RNA. The tRNA remained in the supernatant, and was precipitated and washed using the above described alcohol protocol. The pellet was resuspended in RNase-free water and purity was checked using 260/280 nm absorbance ratio and TBE-Urea PAGE gel. In the case of using TRIzol® (Life Technologies), Trizol was used instead of phenol:chloroform:isoamylalcohol.

Reagent cost calculations were based on previous calculations and on reagent prices obtained from the 2015 online catalogues of Roche Applied Sciences, Sigma-Aldrich, VWR, Zymo Scientific, and Life Technologies [26]. Cost of labor was not included.

3 Results

The protein onconase (ONC) of the RNase A superfamily is a promising biotherapeutic to target multiple forms of cancer. ONC, like other RNases, is difficult to overexpress recombinantly and poses difficulties for overexpression in the endogenous cell-line. Some of the difficulties include cytotoxic activity, ribonuclease activity, and the formation of inclusion bodies. We compare *E. coli*-based in vivo produced ONC (ONC_{invivo}) to CFPS produced ONC (ONC_{CFPS}) in terms of solubility and activity. We demonstrate that CFPS is a valuable platform to rapidly produce and directly screen this biotherapeutic.

3.1 Onconase expression

3.1.1 Recombinant in vivo synthesis of onconase

The ONC gene was designed for optimal T7-promoted *E. coli* expression. The resulting pJ411-Onc plasmid was transformed into BL21-Star™ (DE3) cells. Cells were cultured and induced with 1 mM IPTG 2.5 h after inoculation (OD_{600} 0.4–0.7, Fig. 1A). Control cells harboring the nontoxic pJ411-MbRS (*Methanosarcina barkeri* pyrrolysyl-synthetase) were cultured identically. MbRS was chosen as a control, as it is also known to form inclusion bodies. Induction with IPTG resulted in a premature transition out of exponential phase for both growth types (Fig. 1A). The transition is expected, as energy is diverted from cell growth to protein overexpression and IPTG is a known toxin for *E. coli* [27]. The growth of cells harboring the ONC gene slowed beyond that of the nontoxic MbRS gene (*p*-value = 0.0227), implying the toxic action of ONC may inhibit growth until the toxic protein precipitates or is otherwise inhibited.

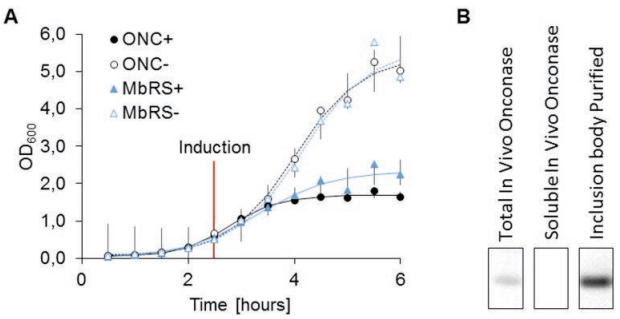


Figure 1. Onconase in vivo expression and solubility. (**A**) Growth rate of *Escherichia coli* harboring plasmids pJ411-ONC or pJ411-MbRS, both genes known to produce inclusion bodies. Growths were induced with IPTG (+) or not induced (–). Graphed curves represent growth regression curves (Supporting information, Table S1). After induction (ONC+ and MbRS+), the growth rate slowed. ONC+ growth rate became nearly static 1 h after induction, which is consistent with the cytostatic/cytotoxic nature of ONC. (**B**) Electrophoretic analysis of total, soluble, and inclusion body fractions of ONC+ growth. The overexpression band is visible only in the total and inclusion body fractions. No soluble ONC was detected.(error bars represent 95% confidence interval of regression).

Previous works report the formation of inclusion bodies during recombinant bacterial expression of ONC_{invivo} [10]. To assess expression and formation of inclusion bodies, crude and centrifugation-clarified lysates were run on SDS-PAGE (Fig. 1B). Protein overexpression bands appear in the crude lysate and not in the clarified lysate, indicating the low solubility of ONC_{invivo}. Indeed, densitometric analysis of the expression bands indicate that less than 1% of the ONC_{invivo} remains soluble (Fig. 1B).

ONC_{invivo} protein with potent nuclease activity was produced only after steps of inclusion body purification, washing and refolding (a three[+] day process, Supporting information, Fig. S3). While the purity of the ONC_{invivo} was greater than 90%, the post-fermentation purification steps are time consuming, expensive, and challenging to scale-up, thus restricting the feasibility of high-throughput screening and inexpensive production scale-up. To overcome the difficulties of in vivo production of ONC, we assessed the effectiveness of a cell-free approach.

3.1.2 *Cell-free synthesis of highly soluble onconase*

ONC was produced in cell-free protein synthesis (CFPS) from the identical plasmid as in vivo and assayed for yield and solubility (Fig. 2A). Initial yields from a standard CFPS reaction were less than 3% of the reference protein Green Fluorescent Protein (GFP) yields (0.03 mg/mL ONC_{CFPS}versus1.45 mg/mL GFP_{CFPS}). However, and notably, greater than 80% of the ONC_{CFPS} yield was soluble, representing a greater than 80-fold increase of percent soluble yield over in vivo expression. If the soluble protein is active, its production would unleash the protein's nuclease activity, likely degrading the machinery used to produce it and leading to lower overall yields. ONC predominantly targets tRNA and can also degrade mRNA and rRNA [28]. We hypothesized that if the soluble product was active, low yields would be due in part to tRNA degradation.

In principle, the inhibition of ONC_{CFPS} during production would improve overall yields by protecting essential RNA machinery for protein synthesis. However, one of the features of RNase A-like ONC is its ability to resist inhibition by RNase A inhibitors, specifically at physiological salt concentrations [29]. This resistance is thought to provide ONC its beneficial anticancer properties, allowing the RNase to function after endocytosis despite the potential presence of RNase inhibitors [23, 30]. Accordingly, when we added a potent RNase A inhibitor (RNase Inhibitor, Murine; New England Biolabs, MA) to our CFPS productions for ONC_{CFPS}, we observed no improvement in overall yields compared to standard CFPS (Fig. 2B, RNase Inhibitor).

3.1.3 *tRNA-supplemented cell-free synthesis of soluble onconase*

We hypothesized that the replacement or supplementation of tRNA would improve overall ONC_{CFPS} yields by mitigating tRNA-degradation effects. The open nature of CFPS makes possible the addition of excess tRNA without need to modify the expression organism. Harnessing this feature, CFPS was performed while providing additional purified tRNA. Addition of an initial bolus of tRNA (96 mg tRNA per mL initial CFPS, dilution with

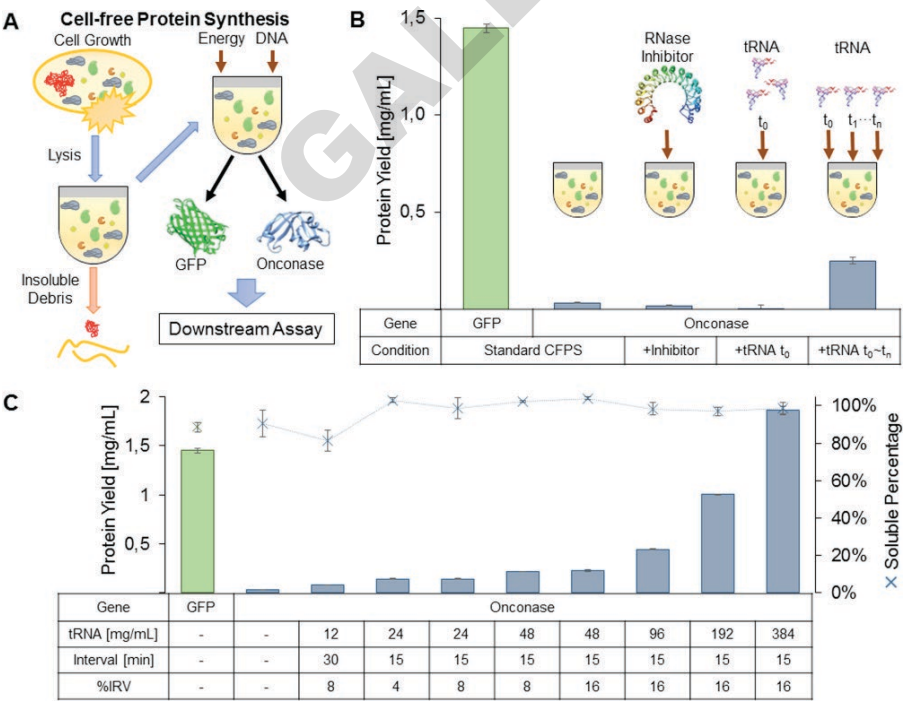


Figure 2. Cell-free expression optimization of onconase. (**A**) Abbreviated scheme of CFPS production for direct downstream applications. (**B**) Modified CFPS methods in attempts to improve ONC_{CFPS} yields by RNase inhibition and tRNA addition. tRNA was added as a single bolus (+tRNA t_0) or dosed throughout the reaction at specific intervals (+tRNA t_0-t_n). (**C**) CFPS optimization of ONC_{CFPS} yields through the addition of tRNA at specified intervals and volumes by percent of initial reaction volume (%IRV). ONC_{CFPS} yields were directly correlated to total tRNA addition per mL of initial reaction volume (tRNA [mg/mL]). Soluble yield percentage (X) were >80% for all ONC_{CFPS}. ($n \geq 3$, error bars represent one standard deviation).

1.92 volumes) effectively eliminated ONC_{CFPS} production (Fig. 2B, +tRNA t_0). One possible cause of this inhibitory effect may be due to excessive deacylated tRNA. Previous work has reported that excessive deacylated tRNA can inhibit binding of amino acyl-tRNA at the ribosome P site [31]. In the case of adding excess tRNA at the start of the reaction, it can be postulated that a majority of tRNA remain non-acylated due to the limited supply of amino acids and aminoacyl synthetases.

As an alternative approach, the same amount of tRNA was added to the CFPS reaction at 15 min time intervals throughout the reaction, resulting in a more than seven-fold yield improvement (Fig. 2B, +tRNA t_0-t_n). To further optimize yields, the system was modified by adding a range of 12–384 mg total tRNA per mL initial CFPS. Stock concentrations of tRNA were 25, 50, 100, or 200 mg/mL in water. Time intervals were every 15 or 30 min. Volumes added at each time interval were 4, 8 or 16% of the initial reaction volume (%IRV). We observed ONC_{CFPS} yield was directly proportional to the amount of tRNA added to the reaction and not effected by dilution within the employed range of %IRV (4–16 %IRV at each interval) (Fig. 2C). For example, yields were statistically indistinguishable when a total of 48 mg/mL CFPS was added, regardless of %IRV.

In the best case, ONC_{CFPS} production increased greater than 56-fold (from standard CFPS of 0.03 to 1.86 mg/mL for the conditions 384 mg total tRNA per mL CFPS, 15 min tRNA addition interval, 16% IRV). This increase in total yield was matched in increased soluble yield. In all cases when reactions were supplemented with tRNA at 15 min intervals, the solubility of ONC_{CFPS} remained above 95% of total yield.

The best ONC_{CFPS} yield outperformed our reference protein (GFP) in standard CFPS by 28%. To verify that standard CFPS isn't limited by tRNA, we performed CFPS with GFP and increasing amounts of tRNA, both with and without dilution. GFP yields did not exhibit statistically significantimprovement with increasing tRNA concentration, indicating tRNA is not a limiting reagent in this case (Supporting information, Fig. S4).

The open nature of CFPS provides the control required to modify the translation environment for ONC_{CFPS} production. The periodic addition of tRNA secures sufficient tRNA for translation processes essential during ONC_{CFPS} expression. Moreover, excess tRNA may act as sacrificial RNA and provide fodder for endogenous *E. coli* RNases and ONC_{CFPS} present in the reaction, aiding in the preservation of essential translational RNA (mRNA encoding ONC and rRNA). Furthermore, adding doses of tRNA throughout the CFPS reaction mitigates any inhibition that excessive deacylated tRNA may have on the ribosome. These effects make our modified CFPS a propitious system for rapid ONC expression.

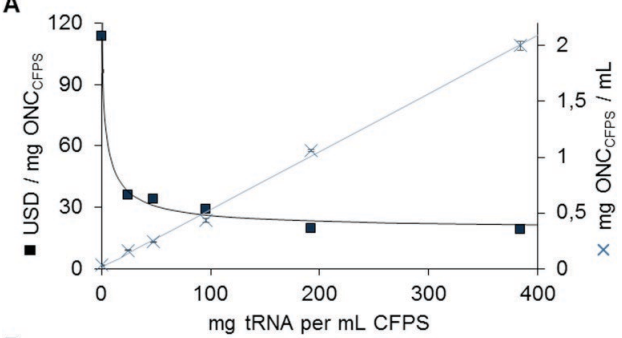
3.1.4 *Scale up of tRNA-supplemented cell-free protein synthesis*

One key component of biotherapeutics is the ability to scale-up production. Our and other's previous works have reported that CFPS is readily scaled, from as little as 15 µL to 100 L [32, 33]. To verify our modified CFPS setup would also scale, we produced ONC_{CFPS} with initial reactions volumes of 50 and 1000 µL. To maintain consistent heat and mass transfer, total reaction volume never exceeded 3 mL in 50 mL conical centrifuge tubes and the tubes were shaken at 280 RPMs. Small and large scale reactions produced ONC_{CFPS} equally well (Supporting information, Fig. S2), indicating our modified CFPS system may work for larger scale as long as reaction engineering principles are considered.

3.1.5 *Cost analysis of tRNA-supplemented cell-free protein synthesis*

While the production of ONC_{CFPS} can be increased in CFPS systems by adding supplementary tRNA, this improvement is beneficial particularly if overall costs per yield decrease. We have previously described the costs of our standard cell-free system [26]. To analyze the impact of tRNA costs due to addition of tRNA, we considered three methods of procuring tRNA: (i) commercially purchased tRNA (Roche, MRE600); (ii) tRNA purified with a standard commercial kit (TRIzol®, Life Technologies); and (iii) tRNA purified with an in-house phenol-chloroform extraction method, detailed in Materials and Methods. Purification costs included all reagents' costs (cost of kit components, cost of consumable materials for cell growth, lysis, and purification). Purified tRNAs were verified to

A



B

tRNA Source	Commercial	Purified	
	tRNA MRE600 Roche	TRIzol® Life Technology	Ph:Chl:IsoAlc In-house
USD/gm tRNA	$1174	$524	**$90**
260/280 nm	2.1	2.0	2.1

Figure 3. Cell-free expressed onconase yield costs. (**A**) ONC_{CFPS} was produced with increasingly concentrated doses of tRNA. Volumetric yields (mg ONC_{CFPS} per mL, x) were directly proportional to the amount of tRNA added. The yield cost (US dollars per mg ONC_{CFPS}, ■) is calculated based on the in-house produced tRNA. (**B**) Table of prices and purities of tRNA sources.($n \geq 3$, error bars represent one standard deviation).

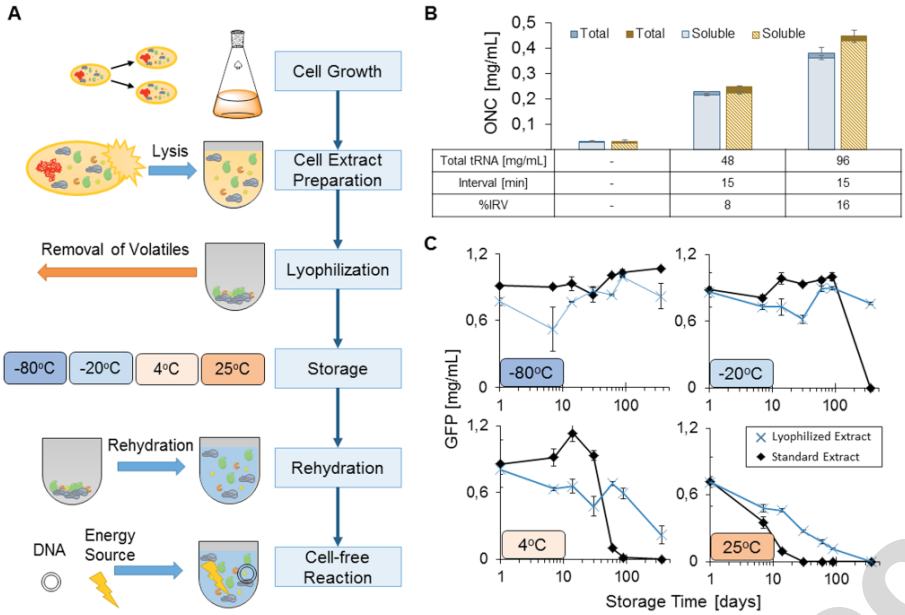


Figure 4. Lyophilized CFPS systems. (**A**) Scheme of lyophilized CFPS system. (**B**) ONC yield comparison between standard aqueous extracts and lyophilized extracts. (**C**) Extract stability data for extracts stored for up to one year under specified temperature conditions. Data up to 90 days was reported previously [15]. The cell-extract utilized for lyophilization was not initially as active as the cell-extract used in Fig. 2, which accounts for the difference in baseline GFP expression levels. ($n = 3$, error bars represent one standard deviation).

have similar or better activity per mg in the CFPS reaction than the commercially purchased material (Supporting information, Fig. S5).

Although convenient and effective, the expense of commercially purchased tRNA outweighed the benefit (Fig. 3B). Indeed, the use of this tRNA increased the costs per mg ONC_{CFPS} by an average of 110% over standard CFPS. Alternatively, the commercially purchased tri-reagent was effective at providing tRNA at about 45% the expense of the commercially purchased tRNA. This price represented a break-even price-point: the average cost per mg ONC_{CFPS} remained nearly at the same level, regardless of tRNA addition. While there is not a yield cost reduction associated with adding commercial or tri-reagent purified tRNA, their addition to the CFPS reactions did significantly improve the yield of ONC per volume, which simplifies and decreases the costs of downstream assays without further treatment.

To further reduce the cost per mg ONC_{CFPS} produced, we developed an in-house developed phenol-chloroform extraction procedure based on the same principles as the commercial tri-reagent. The optimized procedure was effective at reducing the cost of tRNA by greater than 92% compared to the commercially purchased tRNA. The 13-fold reduction in tRNA cost caused overall yield costs to decrease asymptotically from ~115 USD towards ~20 USD per mg ONC_{CFPS} produced (Fig. 3A). This five-fold reduction in ONC_{CFPS} production costs and 56-fold increase in yield was achieved by (i) the direct replenishment of tRNA afforded by the cell-free system and (ii) the development of an in-house optimized tRNA purification method.

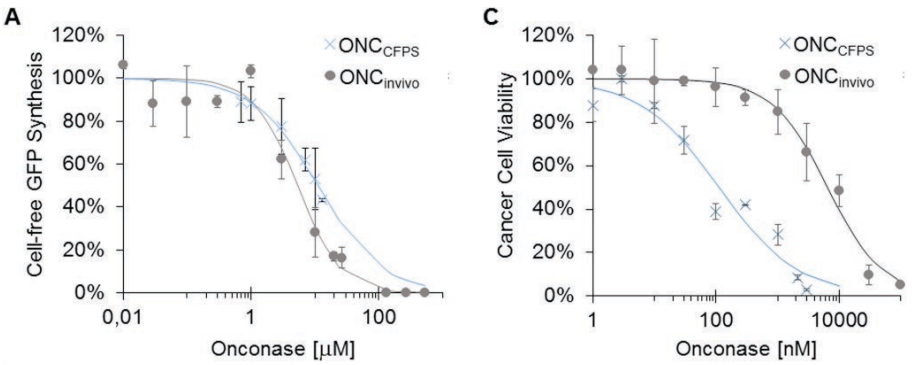
3.1.6 *Lyophilized cell-free system active after one year*

We have previously demonstrated the ability of CFPS to be stabilized up to 90 days by lyophilization, as outlined in the scheme of Fig. 4A [15]. Utilizing this lyophilized CFPS (lyo-CFPS) to produce biotherapeutics is a promising method for rapid, mobile and on-demand therapeutics. The robust stability of lyo-CFPS is characterized by its ability to outperform standard aqueous extracts after storage at temperatures above –80°C (Fig. 4C).

Our previous report included stability data up to 90 days in storage. Fig. 4C reports for the first time the viability of lyo-CFPS stored for one year. The standard extract lost all observable protein synthesis viability under all storage conditions except at -80°C. In stark contrast, the lyo-CFPS retained protein synthesis viability under all storage conditions except after one year at 25°C. We tested our lyo-CFPS system against the standard CFPS to produce ONC_{CFPS}. The lyo-CFPS performed equally well as or better than standard CFPS upon addition of tRNA (Fig. 4B). The robust stability of lyo-CFPS lays the fundamental framework for applications in stabile, mobile, and "just-add-water" biotherapeutic expression.

3.2 Onconase activity

The methods we describe for CFPS production of ONC can reduce costs while providing for direct expression of soluble, accessible proteins ready for downstream characterization and applications. To demonstrate the direct downstream assessment, we analyzed our ONC by; (i) cell-free protein synthesis inhibition assay; and (ii) cancer cell inhibition assay.



B

Protein Synthesis Inhibitory Concentration 50 [IC_{50}]				
ONC_{CFPS}	11.2	±	3.9	μM
ONC_{invivo}	5.3	±	1.1	μM

D

Cancer Cell Inhibitory Concentration 50 [IC_{50}]				
Doxorubicin	0.17	±	0.07	μM
ONC_{CFPS}	110	±	46	nM
ONC_{invivo}	6480	±	1440	nM

Figure 5. Onconase activity assays. (**A**) ONC was added to CFPS of GFP to assay its ability to inhibit protein synthesis. (**B**) Protein synthesis IC_{50} of ONC_{CFPS} and ONC_{invivo}. (**C**) Breast cancer cells (MCF-7) were treated with ONC to assay its ability to inhibit and kill cells. (**D**) Cancer cell viability IC_{50} under treatment with control doxorubicin, ONC_{CFPS}, and ONC_{invivo}. ($n = 3$, error bars represent one standard deviation, IC_{50} ranges represent 95% confidence interval).

3.2.1 *Protein synthesis inhibition activity of onconase*

Protein synthesis inhibition is an indirect measurement of the nuclease activity of ONC and likely plays a key role in the anticancer action. Nuclease activity of cell-free produced ONC_{CFPS} was evident by the increase in protein yield when dosed with tRNA over time. To confirm this effect in controlled conditions, we produced GFP in the presence and absence of ONC. ONC was added at the initiation of CFPS reactions. The addition of ONC_{invivo} resulted in an inhibitory concentration of 50% of protein synthesis (IC_{50}) at 5.3 ± 1.1 μM (Fig. 5A and 5B). The addition of ONC_{CFPS} resulted in an IC_{50} of 11.2 ± 3.9 μM, while negative CFPS controls did not inhibit protein synthesis as severely (Supporting information, Fig. S6). CFPS allowed for immediate downstream analysis of ONC_{CFPS} protein synthesis inhibition.

3.2.2 *Anticancer activity of onconase*

One of the promising characteristics of ONC is its potential as an anticancer therapeutic. To validate our ONC was active against cancer cells, we tested our CFPS and in vivo produced protein against the breast cancer cell line MCF-7 using an MTT cell viability assay, which quantifies the mitochondrial activity after a specified treatment period. As a baseline comparison, we performed the assay with doxorubicin and observed an IC_{50} = 0.17 0.07 μM DOX, consistent with previously published results (Supporting information, Fig. S7) [25].

The treatment of the cancer cells with ONC reduced cell viability by upwards of 95%. The IC_{50} of ONC_{invivo} was consistent with previously published results at 6.48 μM (Fig. 5C, D) [34]. Of considerable note, the IC_{50} of ONC_{CFPS} was about 60 times lower than ONC_{invivo} at 0.11 $_{50}$ of ONC_{CFPS} suggests it may be more potent than ONC_{invivo} or the CFPS reaction mixture may act as an adjuvant for the endocytosis of ONC.

4 Discussion

CFPS production directly to downstream assay (protein synthesis inhibition and anticancer assay) without purification or refolding dramatically reduces the time from gene to meaningful data. This system, together with the ability of CFPS to express genes directly from PCR-produced linear expression templates, lays the foundation for high-throughput technologies to screen large libraries of characterized and uncharacterized proteins for anticancer properties. Such a high-throughput method may reduce research investment of time and money towards future cancer therapies and, in turn, reduce cancer treatment expense to the patient. These CFPS techniques have the potential to also be adapted for other difficult-to-express proteins and biological complexes, such as DNA-protein fusions.

We demonstrated the cell-free protein synthesis system's ability to produce a cytotoxic anti-cancer therapeutic, ONC. We have shown that with inherent open nature of cell-free system, we can freely modify, replace, and supplement the system towards making the final product in a highly soluble and active form without the need of specialized cell lines or complex mutagenesis. With our system, the ONC yield can be increased by 56-fold compared to standard CFPS with greater than 95% solubility. This system can open unique opportunities for making other difficult-to-express biotherapeutics. In addition, CFPS is a promising platform for biopharma high-throughput that can save time and expense, and it can be easily scaled for making commercial or personalized drugs, however cost-effectiveness comparing scaled CFPS to traditional in vivo scale-up would need to be analyzed for each individual biotherapeutics produced.

The robust and stable nature of lyophilized cell-free systems provides further benefit to the production of biotherapeutics. We demonstrated that lyophilized cell-free system was comparable to aqueous CFPS at producing

ONC. Furthermore, lyo-CFPS retains its viability longer under non-ideal storage conditions. Thus, lyophilized extract can enable CFPS to become a platform for mobile biopharma applications, such as lab-on-a-chip, by just adding water to the system.

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