

COMMENTARY



## The growing impact of lyophilized cell-free protein expression systems

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### ABSTRACT

Recently reported shelf-stable, on-demand protein synthesis platforms are enabling new possibilities in biotherapeutics, biosensing, biocatalysis, and high throughput protein expression. Lyophilized cell-free protein expression systems not only overcome cold-storage limitations, but also enable stockpiling for on-demand synthesis and completely sterilize the protein synthesis platform. Recently reported high-yield synthesis of cytotoxic protein Onconase from lyophilized *E. coli* extract preparations demonstrates the utility of lyophilized cell-free protein expression and its potential for creating on-demand biotherapeutics, vaccines, biosensors, biocatalysts, and high throughput protein synthesis.

### ARTICLE HISTORY

Received 6 September 2016  
Revised 21 September 2016  
Accepted 22 September 2016

### KEYWORDS

cell-free; cfps; freeze dry; lyophilize; onconase; protein expression; protein synthesis; tx-tl

### Introduction

Custom protein synthesis is a 50+ billion USD industry that impacts most parts of human lives including the clothes we wear, the food we eat, the beverages we drink, and the lifesaving therapeutics many require.<sup>1</sup> The original workhorse of custom recombinant protein synthesis is *Escherichia coli*, which remains the most cost-effective method and can produce many complex proteins (including insulin<sup>2</sup> and antibodies<sup>3</sup>). Other synthesis chassis including yeast and mammalian cells have been developed for proteins requiring advanced assembly and post-translational modifications such as glycosylation and lipidation.<sup>4,5</sup>

Additionally, cell-free protein expression systems have introduced many attractive advantages to protein synthesis technology.<sup>6</sup> Because the reaction environment is not confined within a cell wall, proteins can be expressed from rapidly-produced PCR gene products,<sup>7</sup> and dynamic optimization of the reaction environment becomes possible.<sup>8,9</sup> Changes in reaction conditions such as redox potential, pH, hydrophobicity, and temperature enable expression of a variety of active proteins that are often difficult to produce *in vivo*. Innovative examples include cytotoxic proteins,<sup>10</sup> membrane proteins,<sup>11,12</sup> metallic holoenzymes,<sup>13,14</sup> and virus-like particles.<sup>15–17</sup> Cell-free protein synthesis (CFPS) reactions also allow the incorporation of unnatural, even toxic<sup>18</sup> amino acids.<sup>19–23</sup>

A major limitation of both *in vivo* and cell-free protein expression methods is the cold storage chain essential for chassis storage. Lyophilizing, or freeze drying, is a common technique for rendering biochemical and bioactive mixtures stable. Lyophilization of wheat-germ extracts and its activity in cell-free protein expression has been reported.<sup>24</sup> Inspired by this report and seeking to overcome scale-up and RNA stabilization challenges of this system, we created a lyophilized cell-free protein synthesis system based on *E. coli* cell extracts for shelf-stable storage and transportation.<sup>25</sup> The system, in addition to breaking the cold-storage chain, has the added benefits of 1) a just-add-water + DNA format where protein can be produced rapidly on-demand – in as little as 1 h, 2) consistent scalable production from 250  $\mu\text{L}$  up to 100 L<sup>26</sup>, and 3) sterilization of the system to prevent release of residual genetically modified bacteria if transported or used in-field.<sup>27</sup>

Thus shelf-stable, on-demand protein synthesis platforms are enabling new possibilities in biotechnology applications. As a particularly innovative example, Pardee et al. have applied lyophilized cell-free systems to enable rapid detection of Ebola and Zika viruses. This cell-free platform was lyophilized into a paper support, and when reconstituted, allowed portable virus detection.<sup>28,29</sup> We recently reported the on-demand production of the cytotoxic cancer therapeutic Onconase

from a lyophilized cell-free protein expression system, which allows for shelf-stable storage of the extract for 90 d before use. The resultant protein was produced at high yields and was immediately available for screening without purification.<sup>10</sup> In this article, we will highlight technological advances and future applications enabled by lyophilized, on-demand protein expression systems.

### **Therapeutic production from shelf-stable expression system: Onconase<sup>10</sup>**

Onconase (ranpirnase) is a potent biotherapeutic that has been used to treat cancer and viral infections.<sup>30,31</sup> Its cytotoxic mechanism of action is the degradation of tRNA, which paralyzes protein production.<sup>4</sup> As a result, high-yield production in living cells is only possible if the protein folds incorrectly into inclusion bodies directly following synthesis. Refolding requires several steps and multiple days to recover some of the original activity.<sup>32,33</sup>

Initial expression of Onconase in *E. coli* cell-free protein synthesis (CFPS) reactions resulted in 80% of produced Onconase being soluble, likely attributed to CFPS's slower expression rates and 25-fold less-crowded environment.<sup>34</sup> However, initial protein yields were less than 3% of those obtained with model protein GFP through *E. coli* CFPS (0.03 mg/mL of Onconase compared to 1.45 mg/mL of model protein GFP). To address the hypothesized tRNA degradation caused by Onconase, tRNA was systematically added to the open CFPS reaction environment. This resulted in an overall Onconase yield of 1.86 mg/mL with more than 95% being soluble. It is also significant to note that the estimated production cost of Onconase is less than 30 USD per milligram.

Following cell-free synthesis, Onconase was added without purification to a breast cancer cell line (MCF-7) and assayed for cell viability. Controls also added to MCF-7 included refolded Onconase expressed in *E. coli*, doxorubicin, and cell-free reagents without Onconase. Cell-free produced Onconase inhibited cancer cell growth 60 times more effectively than refolded Onconase and slightly more effectively than doxorubicin.

In order to demonstrate the enhanced flexibility afforded by lyophilization, Onconase was then synthesized from lyophilized extracts. Onconase yields from lyophilized extracts compared favorably with those from standard extracts, matching or exceeding the standard extract Onconase yields. Taken with our

previous results, which showed extract viability after up to 90 days storage at room temperature,<sup>25</sup> these results demonstrate that a difficult-to-express therapeutic could be produced on-demand, even in remote locations using lyophilized extracts stored at sub-optimal conditions.

### **Future applications of shelf-stable expression systems**

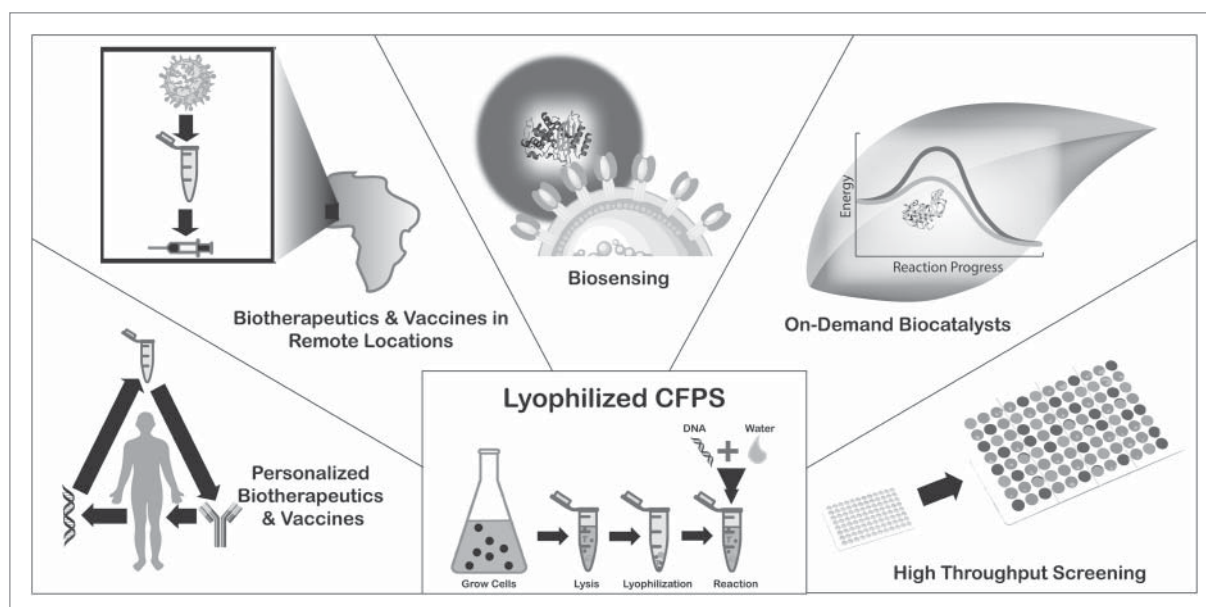
The ability to render protein expression systems shelf-stable enables many exciting applications for cell-free protein synthesis. Among these are personalized and on-demand biotherapeutics and vaccines, biotherapeutics and vaccine production in remote locations, biosensing, on-demand biocatalysis in chemical supply chains, and high throughput protein production for screening, engineering, and protein evolution (Fig. 1).

#### **Personalized biotherapeutics and vaccines**

The process of creating personalized vaccines typically involves isolating nucleic acid from the patient's affected cells in order to express proteins that are then administered to the patient. These proteins take the form of antigens designed to elicit an immune response. Researchers have already demonstrated the creation of personalized therapeutic vaccines for the treatment of lymphoma using cell-free protein synthesis systems.<sup>35,36</sup> Especially in cases of late-stage cancer diagnosis where the time window for effective treatment is severely restricted, rapid and on-demand protein generation is critical. Cancer vaccines have the potential to become increasingly effective and available with the ability to stockpile lyophilized CFPS systems. In addition to creating cancer vaccines, stockpiling of protein expression systems enables rapid vaccine production in response to a viral pandemic threat.<sup>37</sup> Shelf-stable protein expression may additionally impact dendritic cell immunotherapy techniques,<sup>38</sup> enable effective immunotherapy for combating warts,<sup>39</sup> and supply rapid purification-free production of vectors for gene delivery.<sup>40,41</sup>

#### **Biotherapeutics and vaccines in remote locations**

As modern medical treatments depend increasingly on biotherapeutics, the ability to synthesize proteins in remote and adverse environments will be



**Figure 1.** Future applications of shelf-stable protein expression systems. Lyophilized protein expression reactions are activated by the addition of water and nucleic acid, and have the potential to enable innovative applications in personalized biotherapeutics and vaccines, biotherapeutic and vaccine production in remote locations, biosensing, on-demand biocatalysis, and high throughput production.

invaluable. Shelf-stable protein expression systems allow for economic transportation and enable the utilization of biotherapeutics in remote locations. Travelers, humanitarians, and defense units could be outfitted with pre-assembled, just-add-water kits similar to the systems used to produce Onconase. Additionally, lyophilized systems make vaccine production technologies available for generation in remote locations, e.g. in response to an epidemic or in other humanitarian efforts.

### Biosensing

Biosensors are biochemical constructs designed to indicate the presence of a given analyte, and utilize the specificity of a bioreceptor. Biosensor designs that require active proteins in their operations are beginning to be impacted by portable and on-demand CFPS systems. For example, Pardee et al.<sup>28</sup> utilized lyophilization to integrate cell-free protein expression triggered by riboregulators into a paper-based biosensor platform. A chromogenic indicator, visible to the naked eye, was engineered to indicate a positive response from the biosensor. The result was a portable assay, activated simply by adding water and a sample of specific RNA sequence. Applying this previously engineered technology, the authors created a highly specific biosensor for Ebola and Zika. The entire creation process, from *in silico* design to *in*

*vitro* naked-eye detection, required less than 6 d. It is also significant to note that the diagnostic test itself, including sample collection, RNA extraction, and cell-free reaction requires about 3 h to execute, and the lyophilized biosensor platform is shelf-stable for up to one year.

### On-demand biocatalysts

Lyophilized CFPS reactions can also be used to create on-demand biocatalysts, fortifying chemical supply pipelines with increased versatility and economy. Biocatalysts are leading the effort for “green chemistry” in the chemicals industry, eliminating harsh chemical catalysts and waste streams while reducing thermal energy costs. With biocatalysts that can be rapidly synthesized, chemical supply pipelines can be altered in response to a change in the demand of a chemical commodity. Thus chemical feedstock can potentially be more efficiently used to create the chemical product that is most profitable.

### High throughput production

Increasingly rapid gene synthesis technology combined with shelf-stable on-demand protein synthesis is a powerful tool for high throughput protein production. Currently, gene product structure and function understanding lags far behind available gene sequence data. Methods for high throughput and economic

screening of protein gene products are a significant advantage to researchers seeking to decipher the proteome.<sup>42,43</sup> High throughput protein synthesis is also indispensable in rational protein engineering and directed protein evolution. Because standard cell-free extracts must be used quickly or stored frozen, and because preparation of the extracts requires several hours, dependency on such has the potential to bottleneck execution of rapid protein expression cycles. On-demand protein expression systems, that transcribe and translate from rapidly-produced DNA<sup>44</sup> and facilitate activity assessment without product purification, enable parallel synthesis cycles to be executed in as little as 3 to 4 h.<sup>10</sup>

## Conclusion

In an emerging epoch of novel protein systems engineered to meet human needs, technologies providing on-demand protein expression will become fundamentally useful. From cytotoxic cancer therapeutics to Zika virus detectors and on-demand biocatalysts, lyophilized cell-free protein synthesis platforms place exciting new tools in the hands of biotechnologists.

## Abbreviations

CFPS cell-free protein synthesis  
GFP green fluorescent protein

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

## Funding

We would like to acknowledge and thank our funding sources for their generous contribution including the National Science Foundation CBET Division CAREER Award (#1254148) and the National Science Foundation Graduate Research Fellowship Program (Grant #1247046). Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.

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