ESTABLISHING CELL-FREE PROTEIN SYNTHESIS FOR BIOMANUFACTURING

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Introduction. Cell-free protein synthesis (CFPS) has emerged as a powerful technology platform to help satisfy the growing demand for simple and efficient protein production (1-3). While used for decades as a foundational research tool for understanding transcription and translation, recent advances in bacterial CFPS have made possible cost-effective microscale to manufacturing scale synthesis of complex proteins. Protein yields exceed grams protein produced per liter reaction volume, batch reactions last for multiple hours, costs have been reduced orders of magnitude, and reaction scale has reached the 100-liter milestone. These advances have inspired new applications in the synthesis of protein libraries for functional genomics and structural biology, membrane proteins, and protein pharmaceuticals, among others (**Fig. 1**). Two frontier areas of CFPS include the development of cost-effective, high yielding eukaryotic platforms as well as the efficient incorporation of unnatural amino acids (UAAs) into proteins. Here, we will describe our recent efforts in these areas.

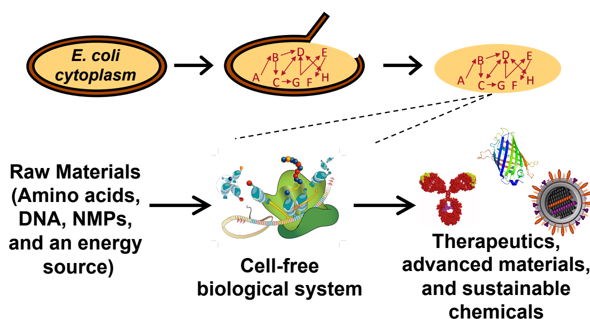

Fig.1: Cell-free biology is the activation of complex biological processes without using intact living cells. Cell-free systems provide an unprecedented freedom of design to modify and control biological systems that is unattainable with traditional fermentation processes.

Methods. Techniques and methods are described in relevant literature (1-5).

Results. Towards the development of a cost-effective, high yielding eukaryotic CFPS platform, we describe recent efforts to create a *Saccharomyces cerevisae* based system (4). *S. cerevisiae* is a natural fit for CFPS because like *E. coli*, it is microbial and can be grown quickly and cheaply under precise conditions in either a bioreactor or shake flasks. Furthermore, it is suited to fold eukaryotic proteins, has facile genetics, and is an important bio-manufacturing production platform. Over the past two years, our team has improved batch protein synthesis yields in a yeast-based CFPS system over two orders of magnitude relative to alternative commonly used methods by making strategic changes to the extract preparation method and combining transcription and translation into a single protein biosynthesis reaction. These advances will be discussed.

Towards the development of efficient incorporation of UAAs into proteins, we describe our efforts to use genomically recoded organisms (GROs) lacking release factor 1 (5). Because the parent strain has not been previously optimized for CFPS, we exploited multiplex advanced genome engineering (MAGE) to design and construct synthetic genomes that upon cell lysis lead to improved extract performance. We targeted the deletion of more than five potential negative effectors (*e.g., rna*), and our approach allowed us to improve protein yields more than 5-fold up to greater than 1g/L. We also catalogued the systems impact of making numerous gene deletions both individually and in combinations. Our work is notable because this is, to our knowledge, the first demonstration of using whole genome editing for extract strain development.

Conclusions. This work will contribute to our fundamental understanding of factors governing cell-free metabolism and play a transformative role in efforts to understand design rules for separating cellular growth (catalyst synthesis) and product synthesis (catalyst utilization). Looking forward, CFPS will facilitate production of protein therapeutics, particularly where cellular toxicity or insolubility problems limit commercially feasible concentrations.

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