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MINI REVIEW

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Cell-free expression systems for rapid prototyping and drug synthesis

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ABSTRACT

Cell-free expression systems have become valuable tools in synthetic biology and biotechnology, offering a rapid and flexible approach to prototyping genetic circuits, metabolic pathways, and therapeutic proteins. By eliminating the need for living cells, these systems provide precise control over biochemical reactions, greatly speeding up the design–build–test cycle in biological engineering. Advances in lysate production, energy supply mechanisms, and modular setups have significantly improved their efficiency, scalability, and range of applications. These developments have opened new possibilities in drug development and manufacturing, including the production of small molecules, biologics, and complex biosynthetic products. Additionally, cell-free platforms are well-suited for studying toxic or unstable enzymes and optimizing pathways that are difficult to explore in vivo. Their compatibility with high-throughput methods and automation further enhances their role in modern biomanufacturing. While challenges such as high costs, limited capabilities for post-translational modifications, and stability issues remain, ongoing progress in engineered lysates, integration with microfluidics, and computational design is continually expanding their potential. This review explores the core principles, recent innovations, and current challenges of cell-free systems, emphasizing their growing impact on drug synthesis and bioengineering.

KEYWORDS

Cell-free expression systems; Prototyping; Drug synthesis; Synthetic biology; Bioengineering applications

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Introduction

The fields of synthetic biology and metabolic engineering have seen rapid growth, driving the need for efficient, adaptable, and controllable platforms to design and test biological systems. Conventional in vivo approaches, though effective, face challenges due to the inherent complexity of living cells, such as regulatory feedback loops, metabolic strain, and the toxicity of foreign compounds. These factors can hinder the pace of the iterative design–build–test processes essential for creating new biosynthetic pathways or therapeutic agents.

Cell-free expression systems (CFES) provide a novel solution by separating protein production and metabolic functions from the constraints of live cells. Utilizing either crude lysates or purified components, CFES create a controlled biochemical environment that allows direct manipulation of DNA, enzymes, cofactors, and metabolites [1,2]. Their open and modular structure supports rapid prototyping, offering fine-tuned control of experimental conditions, real-time analysis, and shortened development cycles.

Ongoing technological progress has expanded the functionality of CFES, making them increasingly suitable for pharmaceutical applications. These systems are now being used for everything from enzyme evaluation and metabolic pathway refinement to the on-demand synthesis of complex drugs [3]. This review examines the key principles, emerging innovations, and real-world applications of CFES, particularly in the context of accelerating the drug discovery and development process.

Overview of Cell-Free Expression Systems

CFES are vital tools in synthetic biology, providing a flexible platform for gene expression and biochemical production outside of living cells. By isolating and assembling the essential components for transcription and translation in a test tube, scientists can precisely control biological processes [4]. This approach is especially useful for fast prototyping, fine-tuning experimental conditions, or producing proteins that are harmful or unstable in living organisms [5].

Types of cell-free expression systems

CFES can be broadly categorized based on the source organism and whether they use crude lysates or purified components.

E. coli-based crude lysates

These are the most commonly used CFES due to their simplicity, low cost, and scalability. Made by lysing E. coli cells, they retain key components for protein synthesis [6]. They offer high yields and are ideal for quickly testing genetic constructs, but lack the ability to perform complex post-translational modifications like glycosylation, limiting eukaryotic protein expression [7].

PURE (Protein synthesis using recombinant elements) systems

PURE systems are built from purified components like ribosomes, tRNA synthetases, and polymerases. They provide a clean, controlled environment ideal for studying mechanisms and engineering gene circuits. Despite lower yields and high costs, they offer unmatched precision and flexibility [8,9].

Eukaryotic cell lysates

Eukaryotic cell lysates from wheat germ, insect (e.g., S. frugiperda), or mammalian cells (e.g., CHO, HeLa) offer a more native environment for expressing eukaryotic proteins. They support disulfide bonds, chaperone folding, and some

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post-translational modifications [10]. These systems are ideal for producing therapeutic proteins and membrane receptors but are costlier and typically yield less than bacterial lysates.

Core components of CFES

All CFES rely on key elements that influence their efficiency:

- **Genetic template:** DNA (plasmid or linear) or mRNA provides the coding sequence for protein synthesis. Linear DNA enables rapid testing, while plasmids offer greater stability.
- T**ranscription machinery:** RNA polymerases like T7, SP6, or E. coli types initiate transcription, depending on the system.
- **Translation components:** Ribosomes, tRNAs, and translation factors assemble proteins from mRNA.
- **Energy Systems:** ATP and GTP are replenished using compounds like creatine phosphate, PEP, or 3-PGA.
- **Amino acids & cofactors:** All 20 amino acids and cofactors (e.g., Mg^{2+}, K^{+}, NAD^{+}, CoA) are added to support enzymatic reactions.
- **Stabilizers & buffers:** Agents like PEG, DTT, and RNase inhibitors maintain reaction stability and prevent degradation.

These components can be fine-tuned to accommodate specific proteins, optimize yield, or study biochemical pathways under controlled conditions.

Table 1. Comparison of major cell-free expression systems.

System Type	Protein Yield	Post-Translational Modifications (PTMs)	Cost	Typical Applications	Advantages	Limitations
E. coli Lysate	High (~mg/mL)	Limited (no glycosylation)	Low	Metabolic engineering, genetic circuit prototyping	Inexpensive, easy to prepare, scalable	Lacks complex folding and PTMs
PURE System	Moderate	None	High	Synthetic circuit design, mechanistic studies	Highly defined, minimal background, modular	High cost, lower protein yield
Wheat Germ Lysate	Moderate	Disulfide bonds, limited PTMs	Moderate	Eukaryotic protein expression, functional studies	Good folding, eukaryotic-like translation	Slower reactions, moderate yield
Insect Cell Lysate	Moderate	Some (e.g., phosphorylation)	High	Membrane protein expression, eukaryotic proteins	Improved folding, eukaryotic environment	More complex setup, higher cost
Mammalian Cell Lysate	Low to Moderate	Complex (e.g., glycosylation, folding)	Very High	Therapeutic proteins, membrane receptor studies	Most authentic folding and PTMs	Cost-prohibitive, batch-to-batch variability

Advantages of Cell-Free Systems for Prototyping

CFES are gaining attention in biological engineering for their simplicity, speed, and flexibility. Free from cellular regulation, they enable efficient design, testing, and optimization of biological parts and pathways [11]. Below are key advantages that make CFES ideal for rapid prototyping and development.

Accelerated design–build–test (DBT) cycles

Traditional in vivo systems face delays from plasmid cloning, transformation, colony screening, and cell growth, which can take days. CFES bypass these steps by directly using PCR-amplified DNA or plasmids, enabling transcription and translation in hours. This speed accelerates hypothesis testing, parameter optimization, and decision-making in pathway design or genetic circuit development [12].

Open reaction system for direct manipulation

CFES provide a controllable, open biochemical environment, allowing real-time addition or removal of substrates, cofactors, regulatory molecules, and synthetic transcription factors [13]. This is ideal for:

- Testing enzyme kinetics
- Modulating metabolite concentrations
- Exploring synthetic feedback/feedforward loops

This flexibility helps metabolic engineers balance fluxes or address bottlenecks in biosynthetic pathways.

Safe expression of toxic or non-natural elements

CFES allow the expression of toxic proteins, non-natural amino acids, and xenobiotic pathways without affecting cell viability. This enables the use of high-yield fluxes and bypasses cellular regulation, transport, or feedback inhibition issues [14].

High-throughput compatibility and parallel processing

CFES can be miniaturized for high-throughput platforms like microtiter plates or microfluidic devices, enabling:

- Parallel testing of multiple pathway variants
- Automated optimization of gene expression conditions
- Fast screening of enzyme libraries or mutant variants

This scalability aids in developing optimized biosynthetic routes for pharmaceutical and industrial applications.

Real-time monitoring and analytical accessibility

With CFES, real-time monitoring via spectrophotometry, fluorescence, or mass spectrometry allows dynamic analysis of metabolite production, cofactor consumption, and enzyme activity, offering more detailed datasets than in vivo cultures [15].

Applications in Drug Synthesis

CFES show great promise in drug synthesis due to their versatility, scalability, and ability to bypass the challenges of

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living cells. They provide a rapid platform for discovering and producing therapeutic compounds, including small molecules and proteins. CFES applications in drug synthesis include enzymatic small molecule synthesis, therapeutic protein and peptide production, and drug-target interaction screening [16].

Enzymatic synthesis of small molecules

Cell-free systems are ideal for enzymatic small molecule synthesis, offering a cleaner, more efficient alternative to traditional chemical methods that can be costly and generate toxic byproducts [17].

Advantages:

- Environmental impact: Reduces hazardous chemicals and solvents, lowering the environmental footprint.
- Customization: Allows rapid tailoring of enzymatic pathways for novel compounds, aiding drug discovery.
- Scalability: Supports scaling from small-scale drug testing to large-scale clinical production.

Examples:

- Antibiotics: Engineered CFPS systems produce penicillin.
- Opioid alkaloids: Morphine is synthesized using enzymatic cascades, bypassing plant-based production.

Production of therapeutic proteins and peptides

Cell-free systems enable faster, higher-quality production of therapeutic proteins and peptides, overcoming challenges like misfolding and low yields in traditional systems [18].

Advantages

- Faster production: Therapeutic proteins are produced in hours, bypassing cell culture maintenance.
- Post-translational modifications: CFPS can perform essential modifications like glycosylation.
- Toxicity bypass: Toxic proteins are easier to produce in controlled cell-free environments.

Examples

- Insulin: Rapid prototyping of insulin forms for testing.
- Monoclonal antibodies: Efficient production for cancer treatment.

Screening for drug-target interactions

CFPS enables high-throughput screening of drug-target interactions, accelerating drug discovery by testing small molecules or biologics on target proteins.

Advantages

- High-throughput: Can screen thousands of candidates quickly.
- Direct, specific assays: Isolated target proteins ensure precise testing without interference.
- Customization: Assays can be tailored to study various drug mechanisms [19].

Examples

- Targeted cancer therapies: CFPS screens small molecules for inhibitors of cancer-related proteins.
- Neurodegenerative diseases: Compounds targeting proteins involved in Alzheimer's and Parkinson's are screened through CFPS.

Recent Advances and Strategies

Cell-free protein synthesis (CFPS) has seen rapid advancements, driven by innovations in synthetic and systems biology, enhancing efficiency and expanding applications in metabolic engineering, regulatory control, and pathway optimization. This section covers two key areas: metabolic pathway prototyping and integration with synthetic biology tools [20].

Metabolic pathway prototyping

Cell-free systems offer a faster, more controllable approach to prototyping metabolic pathways compared to traditional in vivo methods. They bypass constraints like cell viability and feedback inhibition, allowing for open, tunable environment testing [21].

Key advantages

- Rapid iteration: Accelerates the design-build-test-learn (DBTL) cycle.
- Controlled environment: Focuses on optimizing pathways without cellular metabolism interference.
- Real-time analysis: Facilitates monitoring of intermediates and enzyme kinetics.

Case studies

- Isoprenoid biosynthesis: Optimizing pathways for pharmaceutical and fragrance precursors.
- Polyketide synthesis: Prototyping complex compounds quickly, overcoming in vivo limitations.

Integration with synthetic biology tools

CFPS has integrated with advanced synthetic biology tools, enabling dynamic control over gene expression and sophisticated synthetic networks.

CRISPR-based regulation

- CRISPR interference (CRISPRi) and activation (CRISPRa) offer precise control of transcription in cell-free systems, enabling pathway flux tuning [22].
- Example: CRISPRi modulates pathway branches in biosynthesis of aromatic compounds.

Riboswitch and RNA circuit integration

- Riboswitches regulate translation in real-time, and synthetic RNA circuits build logic gates and biosensors [23].
- Useful in diagnostic assays and responsive drug synthesis.

Modular circuit testing

- CFPS allows easy swapping of genetic components for assembling synthetic gene networks, such as toggle switches and oscillators, before cell deployment [24].

Limitations and Challenges

Despite the promise of CFES in research and industry, several challenges remain. One major barrier is the high cost of reagents like nucleotide triphosphates, amino acids, and cofactors, which hinders large-scale production [25]. Lysate preparation is also labor-intensive and lacks batch consistency, while CFPS does not benefit from economies of scale like

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microbial fermentation, limiting its competitiveness in bulk biomanufacturing. To address these issues, strategies such as recycling cofactors, using low-cost extract preparation methods, and utilizing waste biomass for lysates are being explored [26]. Additionally, enzyme stability and cofactor depletion limit the efficiency of CFPS reactions, with degradation from proteolysis and oxidation reducing the reaction window. Solutions like protease-deficient lysates, cofactor regeneration, and encapsulation are being developed to enhance system longevity.

CFPS systems also struggle to replicate intracellular environments, which impacts their ability to perform complex biosynthetic processes. The lack of compartmentalization, essential for substrate regulation and organization, limits the effectiveness of CFPS for certain applications. Post-translational modifications (PTMs) and chaperone-assisted protein folding are difficult to achieve, limiting their use for complex therapeutic proteins. To overcome these challenges, researchers are working on supplementing CFPS with folding chaperones, redox buffers, or PTM enzymes, and developing hybrid or eukaryotic lysates that better support complex protein expression [27]. Additionally, synthetic compartmentalization using liposomes and microfluidics aims to emulate cellular structures and improve performance.

Future Perspectives

The future of CFES is set to advance rapidly, driven by progress in synthetic biology, AI, and biomanufacturing. Key developments include the expansion of CFPS into high-throughput screening (uHTS) through automated platforms and miniaturized systems, enabling rapid drug discovery and comprehensive functional screening. Custom lysate development, using genetically engineered host cells like yeast or CHO, will enhance CFPS for specific applications such as post-translational modifications or protein synthesis. Additionally, AI and machine learning integration will optimize biosynthetic pathways, speeding up the design-build-test-learn (DBTL) process and improving production efficiency. Cross-cutting innovations like portable CFPS kits, synthetic organelles, and sustainable biomanufacturing will further expand CFPS capabilities for use in resource-limited environments, point-of-care diagnostics, and eco-friendly production processes. These advancements promise to revolutionize drug development, biosensing, and the creation of high-value chemicals.

Conclusion

CFES are revolutionizing synthetic biology, biotechnology, and drug discovery by enabling protein and metabolite synthesis outside living cells. These flexible, rapid, and programmable platforms facilitate the design and testing of biological components, from genetic circuits to therapeutic compounds. CFES overcome limitations like toxicity and expression bottlenecks, and integrate with advanced tools such as CRISPR, riboswitches, and RNA circuits. Although challenges like cost, stability, and cellular complexity remain, innovations in lysate engineering, energy regeneration, and artificial compartmentalization are rapidly advancing the field.

Looking ahead, CFES are moving toward high-throughput automation, customized lysates for specific biochemical tasks, and AI-guided workflows to enhance the design-build-test-learn cycle. These developments position CFES as not just a prototyping tool, but as a future cornerstone for distributed, sustainable, and intelligent biomanufacturing. As the technology advances and integrates with automation and computational tools, CFES will open new frontiers in drug synthesis, diagnostics, biosensing, and personalized medicine.

Disclosure statement

No potential conflict of interest was reported by the authors.

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