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The Emerging Age of Cell-free Synthetic Biology

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Abstract

The engineering of and master over biological parts has catalyzed the emergence of synthetic biology. This field has grown exponentially in the past decade. As increasingly more applications of synthetic biology are pursued, more challenges are encountered, such as delivering genetic material into cells and optimizing genetic circuits *in vivo*. An *in vitro* or cell-free approach to synthetic biology simplifies and avoids many of the pitfalls of *in vivo* synthetic biology. In this review, we describe some of the innate features that make cell-free systems compelling platforms for synthetic biology and discuss emerging improvements of cell-free technologies. We also select and highlight recent and emerging applications of cell-free synthetic biology.

Keywords

- Cell-free
- Synthetic biology
- Cell-free synthetic biology
- Cell-free protein synthesis
- *in vitro*
- Gene circuits

Highlights

- Discuss key features and benefits of a cell-free approach to synthetic biology
- Emerging advancements of cell-free systems for improving synthetic biology
- Cell-free for pharmaceutics, gene circuits, biocatalysis and unnatural amino acids

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1 INTRODUCTION

Thanks to the shoulders of many biotechnology giants, we are now leaving the era of scientific discovery and entering the era of scientific mastery. The development of recombinant DNA tools, the advent of rapid, inexpensive sequencing technology, and now the development and stockpiling of BioBricks has endowed biotechnologists unprecedented speed and power to master the genetic framework of an organism [1, 2]. Indeed, researchers can completely replace genomes or make site-specific rationally designed genome modifiations in only a few days [3-5]. This mastery has sparked the paradigm changing field of synthetic biology.

Synthetic biology (synbio) is considered the engineering discipline of biology and first began when humans sought to manipulate biology toward a desired product. Modern day synbio has its roots in the development of recombinant DNA tools and has exponentially grown in the 21st century (Figure 1). Currently, products of genetically modified cells comprise ~2% of the US economy (~$350Bil USD/year) [6]. Exciting new commercial applications of engineered synthetic biochemical pathways include economically produced anti-malarial pharmaceutics, petroleum-like biofuels, and insecticides [7-9].

Increasingly elegant and powerful tools are being developed to streamline genetic engineering for synbio applications. Examples of these tools include MAGE and Cas9-CRISPR technologies for rapid directed genome editing [3-5], the development of diverse well-characterized BioBricks (gene parts/circuits) [1], and the synthesis of entirely synthetic viable genomes [10]. There are, however, fundamental limitations to an *in vivo* synbio approach. These limitations are innate to the living organism that hosts the synthetic pathway and include:

- Control of the gene circuits and chassis behavior
- Monitoring of synthetic pathway dynamics
- Automation and high-throughput optimization
- Economic expansion into low margin applications
- Efficient and broadly applicable chassis systems

An attractive approach to address these challenges is the emerging and exponentially growing field of cell-free synbio (CF-synbio) (Figure 1). CF-synbio uses an *in vitro* approach to overcome the inherent limitations of using a living organism. A number of reviews have recently sought to address the transformative far-reaching potential of CF synbio [11-13]. This review focuses on how CF synbio can address the challenges of *in vivo*-based synbio listed above. We also describe the impact of CF synbio on protein synthesis related applications such as pharmaceutics, biocatalysis, genetic circuit design, and unnatural amino acid incorporation (Figure 2). The unique advantages of a CF approach demonstrated in increasingly diverse and potent applications set the stage for a golden age for CF synbio.

2 ENGINEERING CELL-FREE SYSTEMS FOR SYNTHETIC BIOLOGY

The most distinct feature of CF systems is the openness of the reaction environment. The open access afforded CF allows for propitious features, such as direct control of regulatory elements, addition of cofactors and enzymes, and *in situ* monitoring. Further benefits of CF are real-time control, simplified metabolic load balances, reduced toxicity effects. These features make CF a compelling platform for developing synbio applications.

2.1 CONTROL

A functional synthetic pathway is dependent on appropriate gene circuit design. *In vivo* systems must achieve metabolic load balance to avoid cytotoxicity, maintain cell growth, and optimize product yields. The ability to appropriately regulate genetic circuits *in vivo* is impeded by our limited ability to predict the behavior, kinetics, and interactions of these circuits [6, 12]. More specifically, variability of gene expression is introduced by each of the individual gene parts, such as promoters, ribosome binding sites, gene sequence, and vector origin of replications; all of which can impact expression levels in ambiguous manners [14]. Optimizing synthetic pathways is further complicated by idiosyncratic and often cumbersome gene delivery methods [6]. These impediments add time, expense, and complexity to creating and optimizing viable synthetic pathways; as illustrated by Jay Keasling Group's triumph to synthetically produce artimisenen in yeast, which took an estimated 150 person-years to achieve [7].

As synthetic pathways become more complex, the need to predict the behavior of individual circuits and elements increases. Although there have been strides in recent years towards improving predictive analysis, state of the art remains insufficient to accurately predict the behavior of all genetic elements in concert. CF-synbio is capable of simplifying and avoiding many of these issues and may be key to unlocking their secrets for future synbio applications.

2.1.1 Gene Regulation

Although gene expression remains subject to variability from its genetic elements, CF-synbio systems can overcome the challenge of controlling gene expression levels by allowing the user to directly manipulate gene template concentration. Expression levels are positively correlated with template concentration [15, 16]. Furthermore, precise control can be directly exerted over the level of RNA polymerases and even ribosome content [17, 18]. Therefore, in CF-synbio, gene design can be simplified to a single cassette type (eg – T7 promoter/terminator), allowing for high-throughput optimization of enzyme and cofactor balances [19]. Recent potent examples of facile control through template concentration include optimizing synthetic tRNA content [15, 20], balancing protein expression for multi-protein assemblies [16, 21, 22], chaperone protein content [23-25], coexpressed immunosorbance assays [26], and *in vitro* reconstitution of eukaryotic translation factors [27].

A further concern of effective synbio design is the characterization of individual regulatory elements. The capacity to directly control gene content in CF-synbio systems also makes them ideal chassis for identifying the individual regulatory effects. CF permits rapid prototyping of regulatory elements, in some cases reducing characterization time more than 5 fold over *in vivo* [28, 29]. Coexpressing multiple elements in CF-synbio provides finer insight of direct interactions while reducing the impacts of metabolic load imbalances or uncharacterized interactions that burden *in vivo* analysis [20, 30, 31]. The potential to more accurately characterize regulatory elements lays a foundation for more accurate modelling and design of gene circuits [32-35]. Increased comprehensive knowledge of circuit parts provides details essential for more accurate computer aided design (CAD), a much needed tool for the synbio community [7].

2.1.2 Chassis Regulation

The intracellular environment of heterologous host chassis inherently differ from the native environment of the synthetic parts. Dissimilarities can lead to undesirable consequences such as inclusion bodies, improperly folded proteins, metabolic load imbalances, and toxicity to the host chassis [7, 36, 37]. Therefore, host organisms may require unique genetic and fermentation engineering for optimal expression of each synthetic pathway, adding labor and cost intensive steps to optimization [38].

Just as the open nature of CF systems allow for dexterous manipulation of the genetic template content, many other aspects of CF chassis may be freely controlled and optimized. Components of the CF-chassis can be directly added, removed or inhibited including nucleic acids, proteins, substrates, cofactors, and chaperones [39-41]. Furthermore, other important features of the reaction environment may be optimized for individual reactions such as the redox potential and pH [23, 42]. The precise and agile control made possible by CF-synbio reduces or eliminates the need to engineer a unique chassis for each synthetic pathway [43, 44]. Furthermore, CF-synbio may be used to identify and overcome complicating aspects of a potential chassis before implanting the synthetic pathway into the host [45].

2.2 MONITOR

Rapid *in situ* monitoring of synthetic pathways is essential for rapid engineering. Current techniques for real-time *in vivo* monitoring rely heavily on coexpressed fluorescent reporter proteins with its additional layer of metabolic load and potential unintended interactions [6]. Other monitoring techniques, such as mass spectrometry or RT-PCR, are insufficiently timely to be considered for rapid or *in situ* monitoring. In general, *in situ* monitoring *in vivo* has limited value as cells innately impede access to the intracellular reaction environment, restricting *in situ* control possibilities to fermentation condition adjustments.

CF-synbio expands the available tools for *in situ* monitoring beyond fluorescent protein expression. Examples include bioluminescence, FRET, and aptamer-based binding assays [46, 47]. One elegant approach made possible by CF was recently demonstrated by Rendl and coworkers, who employed microfluidic reactors with the capability of online real-time sandwich tagging assays [47]. Similar to *in vivo, in situ* monitoring of CF systems relies heavily on spectrophotometry. The development of non-spectrophotometric monitoring techniques will facilitate more diverse applications. Nonetheless, monitoring CF-synbio systems provides the capacity to immediately and directly respond to system dynamics, opening the doors for much needed *better,* rather than *more,* design [6]. The combination of real-time monitoring and real-time control makes it possible for CF-synbio to incorporate external process control engineering into the systems dynamics [34].

2.3 AUTOMATE

The open and modular features of CF-synbio make it a propitious platform for parallel or sequential rapid prototyping. Automation using liquid handlers has enabled parallel screening of microliter reactions that produce sufficient yield for miniature biophysical assays, reducing production and analysis from days to hours [48] [49]. Recent strides in microfluidic technologies provide ultrahigh-throughput mechanisms using *in vitro* compartmentalization (IVC). Femptoliter microfluidic reactors, such as emulsions or plugflow modes, have been utilized for synthetic cell facsimiles [50-52], online aptamer assays [47], probing *in vitro* expression noise [53] and probing complex genetic circuits at steady state [34]. IVC reactions have enabled 10^6 genes to be analyze with just 150 μL of reagents [54]. Employing IVC, researchers have been able to analyze up to 2000 individual reactions per second, allowing for 10^6 genes to be assayed in well under an hour [54]. Using IVC for accelerated *in vitro* evolution minimizes or entirely avoids idiosyncratic steps of *in vivo* systems such as cloning, transformations, and gene recovery [45, 55].

2.4 ECONOMICS

CF systems are generally more expensive than *in vivo* production per gram of protein with approximately 50% of the cost of CF systems associated with the required energy source [40]. CF systems typically rely on expensive high-energy metabolites (e.g. phosphoenolpyruvate, creatine phosphate) as the primary

energy source. In contrast, *in vivo* systems can rely on significantly less expensive and more energy-rich molecules for ATP regeneration (e.g glucose, tryptone). Thus, an attractive technology would be CF energy systems that utilize more energy-rich metabolites. Considerable strides have been made to improve energy sources [56-58]. In a notable example, Caschera and Noireaux recently reported an improved energy system that incorporates maltose in the metabolic pathway and results in upwards of 2.3 mg per mL in a simple batch reaction [58]. Increasingly robust and inexpensive energy sources will be essential for CF-synbio research to realize its full potential.

An emerging benefit of CF-synbio is the movement to reduce costs involved with analyzing and optimizing synbio. For example, screening genetic parts using the IVC in lieu of plates can reduce overall volume required to screen approximately 78 000-fold [54]. For a screen of 10^6 genes, this would lower the costs of reagents from $2.4 million USD to about $31 USD. As the reaction volumes decrease, yields of CF systems have continued to increase. Continued efforts in cost reduction are being pursued such as the alternative reaction mode of continuous exchange CF (CECF) [34, 48]. Reducing the costs of CF-synbio will further expand its utility to diverse low margin applications, such as biofuels and industrial biocatalysts [59, 60].

2.5 EFFICIENCY

There is rich diversity of CF systems, reaction modes, and preparation techniques that provide many options for applying CF synbio. For example, there have been considerable well-described iterations towards streamlining preparation of *E. coli*-based CF extract in the last 30 years [61, 62]. Some preparations also facilitate special applications, such as S150 extract this is free of ribosomes [17]. A lingering difficulty of preparing extracts is consistency of extract viability, which can be affected by cell growth rate, harvest time, gene content, media composition, and growth temperature. We have made recent steps towards optimizing these variables on individual systems, yet general fundamental understanding of the impacts of these variables is not fully characterized [24].

Myriad CF platforms have been reported based on various organisms (e.g. *E. coli, Spodoptera frugiperda*) and various strains of given organisms (e.g. *E. coli* K12, *E. coli* BL21). Platforms are being continually developed such as recently developed platforms from *Saccharomyces cerevisiae* [29, 62] and human hybridomas [63]. Unfortunately, only a limited subset of the platforms reported in literature are commercially available. However, with continued development these diverse platforms will provide a greater variety of chassis to select from in the future and may further streamline optimization of a CF-synbio system.

The open nature of CF allows users to employ the most efficient mode of reaction, whether it be batch, semi-batch, continuous exchange or continuous stirred tank reactors [64, 65]. Unlike *in vivo* systems that are generally confined to the cell, CF synbio reactors can be optimized for each synthetic pathway. Using reactor design, CF synbio reactors can be aligned in sequence or parallel [52], perform coupled and uncoupled reactions [66], and be of any size and shape for optimal performance [52, 67, 68]. Indeed, reactor design principles can be directly applied to CF-synbio reactor engineering. Toward this end, CF-synbio reactors have already been successfully scaled up to 100L [69] and the components necessary for CF synbio reactions can be stabilized for long-term storage and stockpiling [70].

3 EMERGING APPLICATIONS OF CELL-FREE SYNTHETIC BIOLOGY

Although CF-synbio is a newly emerging field, its utility has already been demonstrated for a number of diverse and exciting applications, as detailed in previous reviews [12, 13, 71, 72]. Below, we highlight a sampling of recent applications of CF synbio for protein production.

3.1 PHARMACEUTICALS: ANTIBODIES

CF-synbio systems have been demonstrated to be advantageous for antibody production. For example, in the production of anti-HER2 antibodies and antibody fragments, molecular chaperones (yeast and *E. coli* protein disulfide isomerases) were added to facilitate active protein production up to 300 mg/L using a prokaryotic CF system [25]. Furthermore, the direct addition of glutathione buffers enabled optimization of the redox conditions to facilitate proper formation of up to 16 disulfide bridges per antibody [25, 73, 74]. CF-synbio also enables gene expression of multiple products that can be easily tuned to facilitate proper product interactions. This principle was demonstrated by Yin and coworkers, where light chains was solely produced for one hour before adding the plasmid to produce complementary heavy chains [25]. This sequence prevented the aggregation of the heavy chain proteins that naturally occurs in the absence of sufficient light chain proteins [25]. In addition to prokaryote-based CF systems, eukaryote-based systems are also being investigated for the production of antibodies. Researchers are currently engineering endoplasmic reticulum-derived membrane vesicles to facilitate correct folding and posttranslational modifications such as glycosylation [74, 75].

3.2 PHARMACEUTICALS: VACCINES

Using CF-synbio, the opportunities for vaccine innovations and development are expanding. The primary advantage of a CF system is the ease of optimizing both the reaction and the product stability by adjusting DNA template concentrations and controlling the redox potential for optimal disulfide bond formation [22, 76, 77]. This control has enabled researchers to rapidly produce personalized vaccines for Lymphoma [76]. Another advantage of the CF system is the ability to recombinantly express toxic proteins that would inhibit *in vivo* expression [78, 79]. This ability is improving vaccine development capabilities, particularly in malaria research [78, 80]. Malaria research has been greatly hindered by the inability to produce sufficient protein *in vivo* due to the high A/T content within the genes. For example, attempts to recombinantly express 108 malarial proteins in *E. coli* cells resulted in only 60% being successfully produced [80]. A further challenge for *in vivo* production is the potential to incorrectly glycosylate the immunogenic proteins, which can promote an incorrect immune response [80]. Using CF synbio, two different research groups have overcome these challenges. Doolan and coworker employed an *E. coli*-based CF system supplemented with rare transfer RNAs to facilitate translation of A/T rich regions, resulting in >90% efficiency in expression of 250 malarial proteins [81]. Tsuboi and coworkers were able to express 478 of 567 malarial proteins by employing a wheat germ-based CF system, which avoids glycosylation of the proteins because it lacks the glycosylate enzyme [80]. These CF-synbio systems are capable of producing the proteins necessary for rapid vaccine screening.

Virus-like particles (VLPs) are compelling platforms for vaccines as they are virus-like while remaining noninfectious and the polyvalent nature of VLPs increases immunogenicity [82]. While *in vivo* production of VLPs has existed for years, there have remained obstacles to efficient production such as cytotoxicity and control over expression of monomer proteins [82].

Using CF synbio, rapid optimization of more robust and stable VLPs is possible [83]. For example, CF synbio has facilitated co-expression of cytotoxic proteins for VLP formation [79], controlled disulfide

bond formation for VLP stabilization [42, 79, 84] and controlled uAA incorporation [84]. A particularly well-designed application of CF synbio for vaccine development incorporated uAAs in bacterial flagellin that were subsequently immobilized on VLPs [85]. Using the features of CF-synbio, cofactors and reagents were rapidly optimized to improve CF yields of flagellin from 263 ug/mL to 336.3 ug/mL, [85]. The VLP-immobilized flagellin exhibited a 10-fold increase in bioactivity compared to free flagellin, laying the foundation for a new generation of super-vaccines [85].

3.3 BIOCATALYSIS

Use of CF synbio in biocatalysis enables production of complex biocatalysts. For example, [FeFe]-hydrogenases have some of nature's fastest hydrogen production rates but are poisoned by oxygen and require multiple specialized chaperones to assemble and deliver the [FeFe] biocatalytic core to the hydrogenase protein [86]. Using CF-synbio, Boyer, Kuchenreuther and coworkers produced these hydrogenases in an anaerobic environment and in the presence of the appropriate molecular chaperones, leading to successful assembly of the [FeFe]-hydrogenases [86, 87]. This tool provided sufficient insight to decipher assembly mechanisms of the organometallic biocatalytic core [88, 89]. In another example, researchers have included chaperones to increase production and appropriate folding more than 5-fold of the industrially relevant enzyme *Candida antartica* lipase [24, 90].

By mimicking biological pathways, combining portions of different pathways, and controlling the proximity of different enzymes, CF systems have been used to develop new reaction pathways which rapidly transfer reactants through an enzymatic sequence to manufacture a product [11]. For example, in production of hydrogen from glucose, a CF synthetic enzyme pathway allowed for a 3-fold increase above the theoretical maximum production in microbes [91].

3.4 GENETIC CIRCUITS

The development of modular gene circuits is a vital tool in the synthetic biologist's toolbox because it allows for quick assembly and control of novel complex networks [30, 92]. Progress has been made with *in vivo* circuitry [93-95]; however, *in vitro* methods provide unique advantages including 1) control and predictability of the reaction environment [96-98], 2) a number of well-characterized components allowing for expedited prototyping [92, 99], and 3) accessibility to the reaction that makes *in vitro* circuits truly modular [92, 98-100]. A powerful example of the benefit of *in vitro* is the development of a rapid prototyping cycle that can be performed in a standard business day. This allowed for the prototyping of a 4 component genetic switch in under 8 hours [92].

Taking advantage of the rapid prototyping cycle available from CF systems, many CF circuits have been engineered including logic gates, memory elements, and oscillators. Specifically, researchers have engineered diverse logical gates including AND, OR, NOR, XOR, NAND, and NOT [19, 30, 100, 101]; memory elements such as 1-input "push-push" and 2-input switches [35, 96, 102, 103]; and numerous oscillators [34, 99, 104]. A particularly attractive application of these CF genetic circuits is the engineering of minimalistic artificial cells and cell-like microdevices. An impressive example of this is the demonstration of an oscillator in microemulsion droplets ranging from 33 femptoliters to 16 picoliters [104]. This small partitioning induced broad variability in the initial reagent concentrations within each microemulsion, in effect producing a richly diverse population of reactions [104]. This induced variability could be used to produce large data sets in an ultrahigh-throughput manner for the characterization of non-linear biochemical networks and parameter estimation for circuit behavior.

3.5 UNNATURAL AMINO ACID INCORPORATION

Unnatural amino acid (uAA) incorporation is another powerful synbio tool that allows for unique residue chemistry to be incorporated into proteins. This technology has myriad promising applications such as ligand-protein interaction, biotherapeutics, and biocatalysis [105-107].

There are two predominant approaches to incorporate uAAs. First, the global residue replacement method utilizes natural amino acid analogues that are recognized by the native machinery and replace the natural amino acid counterparts [108]. This requires an auxotrophic host and is limited to a specific subset of uAAs that are sufficient analogs. Second, codon reassignment or stop-codon suppression methods rely on mutated aminoacyl-tRNA synthetase/tRNA pairs (aaRS/tRNA) that orthogonally aminoacylate and deliver uAAs for incorporation [109]. CF synbio has been successfully applied to both approaches. For example, CF global residue replacement eliminates the need for an auxotrophic host and allows for easy optimization of uAA concentrations to avoid bottlenecks at the uAA incorporation site [84]. The CF synbio paradigm has also provided marked improvements to stop-codon suppression, including progressively improved yields [110, 111], optimization of tRNA/aaRS pair and uAA concentrations [15], real-time measurement of reaction conditions [20], and deprotection of uAA *in situ* [112]. A particularly elegant example of CF synbio improving uAA incorporation is the control of orthogonal tRNA (otRNA) content using coexpression in a CF system [15]. This coexpression increased yields by providing higher concentrations of otRNA while circumventing previous labor-intensive steps of otRNA expression and purification. The simultaneous production of otRNA also eliminates the need for modified cell strains for *in vivo* production of a particular otRNA and allows for convenient, rapid incorporation of any uAA with a single standard extract.

This ability to site-specifically introduce novel chemistry into proteins using CF-synbio opens up many diverse applications including controlled drug attachment to antibodies for homogenous production of therapeutics [105], orientation-controlled covalent immobilization of proteins to non-biological surfaces [113], functionalized virus-like particles [114], and incorporation of probes and labels [106, 115]. A poignant illustration of the usefulness of CF uAA incorporation is the site-specific incorporation of PEGylated amino acids into protein therapeutics to prolong stability, and thus lifetime, in the bloodstream [116].

4 CONCLUSIONS

In the biological engineering field of synthetic biology, cell-free systems are emerging as a powerful tool to overcome inherent limitations of living cells. Specifically, the open nature of CF systems enables unparalleled control of gene expression, chassis optimization, *in situ* monitoring, and automation. In addition, CF systems are becoming increasingly attractive for more applications due to recent developments that reduce the cost and improve the efficiency of CF. Already, CF-synbio has been demonstrated as an effective tool for the applications of antibody production, vaccine assembly, gene circuit development, biocatalyst production, and uAA incorporation. The growing number of applications and innate advantages discussed above set the stage for an emerging age of cell-free synthetic biology.

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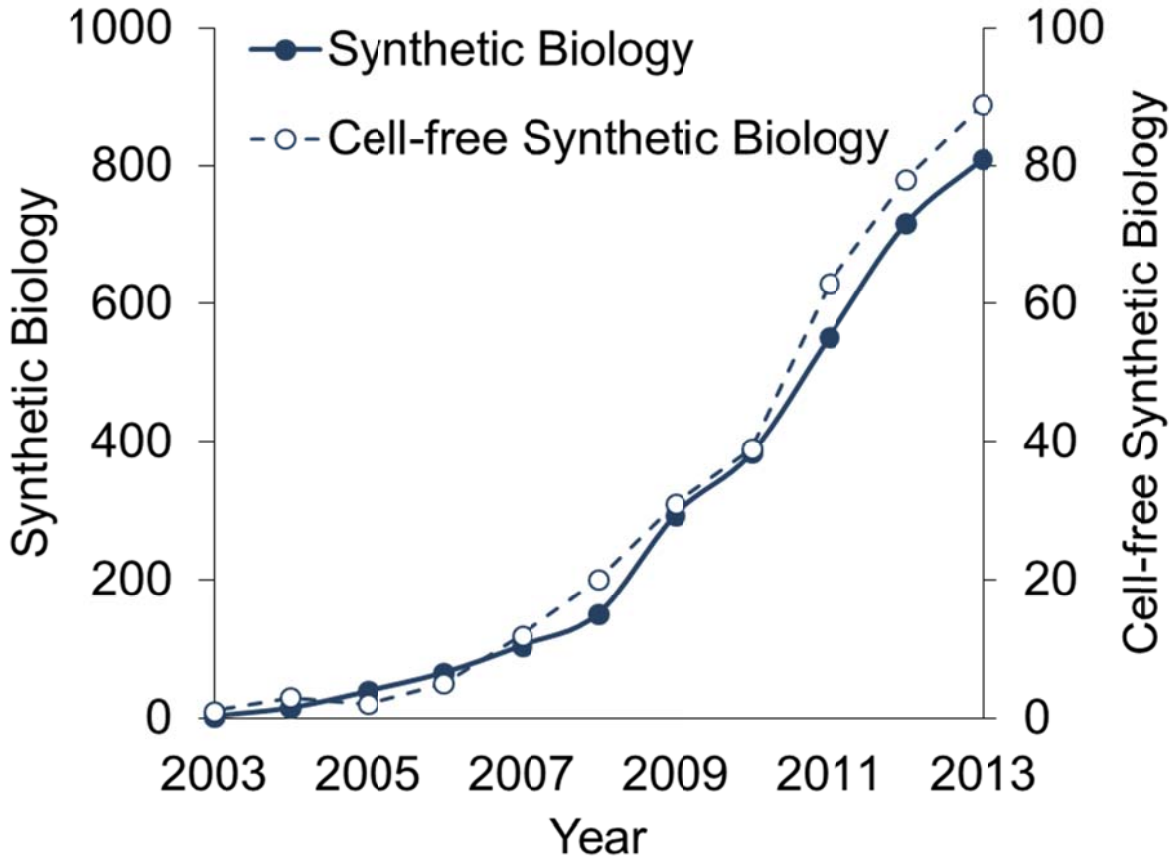
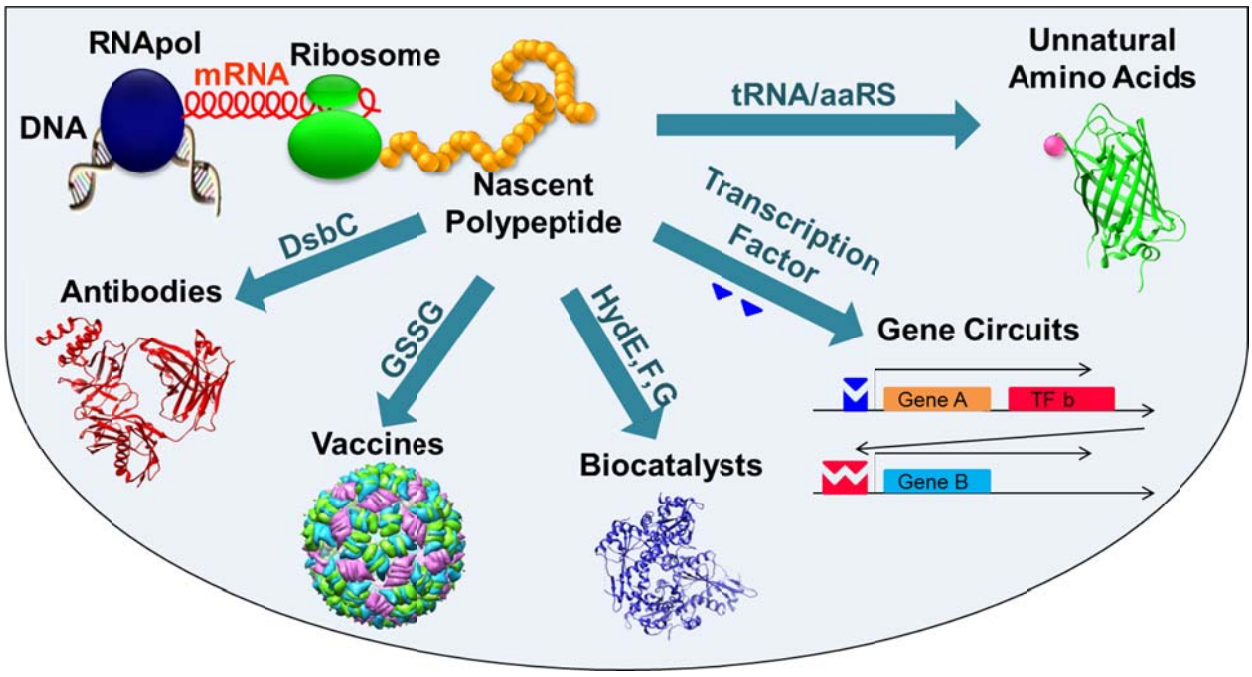
7 FIGURES AND CAPTIONS



7.1 FIGURE 1 – PUBLICATIONS PER ANNUM FOCUSED ON SYNTHETIC BIOLOGY AND CELL-FREE SYNTHETIC BIOLOGY.

Research publications focused on synthetic biology and cell-free synthetic biology have both grown exponentially during the past decade. Data obtained from Thomson Reuters Web of Knowledge journal database.



7.2 FIGURE 2 – HIGHLIGHTED CELL-FREE-SYNTHETIC BIOLOGY APPLICATIONS IN PROTEIN SYNTHESIS

Illustration of the CF-synbio applications in protein synthesis that are highlighted in this review. The open environment of CF systems allows for direct addition and optimization of necessary chaperones and cofactors (specified for each application in the text above the arrows). DsbC represents the disulfide bond isomerase from *E. coli*. DsbC can be optimally coexpressed or exogenously added to CF-synbio reactions to facilitate the proper formation of disulfide bridges in antibodies. GSSG represents oxidizing glutathione buffers to the control redox potential. HydE, F, G represents the molecular chaperones required for proper assembly of [FeFe]-hydrogenases. These chaperones can be optimally coexpressed or exogenously added to CF-synbio reactions to produce the di-iron catalytic core. Transcription Factor represents an input to initiate a genetic circuit or oscillator. For CF-synbio, these can be exogenously added or coexpressed in the reaction. The open nature of the system allows for virtually instantaneous induction or inhibition of transcription factors, allowing for improved characterization of individual and complex genetic elements. tRNA/aaRS represents the orthogonal tRNA and aminoacyl-tRNA synthetase pairs required for site-specific incorporation of unnatural amino acids. These tRNA/aaRS pairs can be exogenously added or coexpressed with the protein of interest. Additional applications of CF-synbio beyond the protein-synthesis-focused applications illustrated can be found in other excellent reviews [12, 13, 71, 72].