Applications of synthetic biology in drug discovery

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Abstract: The applications of synthetic biology have expanded rapidly in the past decades, thanks to advances in DNA synthesis, gene sequencing and lower costs, as well as rapid advances in genomics and data science. Synthetic biology can be used to design new biological systems, or redesign existing systems to implement properties and new functions that humans need, for applications as diverse as disease diagnosis, manufacturing, agriculture and medicine. In the pharmaceutical sector, the process of traditional drug discovery is time-consuming, expensive and challenging, with a low success rate. High expectations have been placed on how to apply synthetic biology in drug development to improve the efficiency and success rate of drug development. In this review, the discovery of new natural products, verification of targets, large-scale drug production and the specific application of synthetic biology in the field of drug research and development are discussed, and the application prospects and existing problems of synthetic biology technology in drug research and opening are prospected.

1. Discovery of new natural products

Natural products are compounds produced by organisms in nature that possess pharmacological or biological activity. They serve as important sources of drugs and drug lead compounds. According to statistics (Figure 1), one-quarter of drugs approved for market from 1981 to 2019 originated from natural products and their derivatives [1]. Natural products have made significant contributions to the treatment of numerous diseases, such as artemisinin for malaria, simvastatin for lowering cholesterol, paclitaxel for cancer treatment, and antibiotics like penicillin and erythromycin.

Despite their ability to inspire new drug development, natural products have faced challenges in the 21st century due to issues such as low accessibility and high development costs, hindering their use in pharmaceutical development.

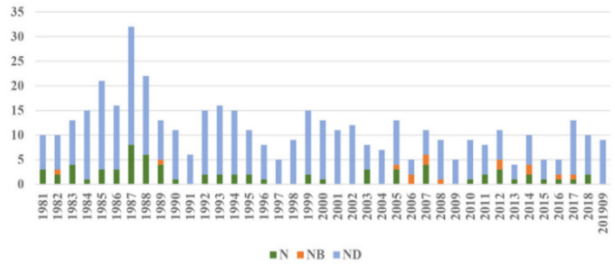

Figure 1. Statistics of Drugs Approved for Market from 1981 to 2019. Reference: Same as Reference [1]

1.1 The problems with traditional methods of natural product discovery

Novel natural products have the potential to break through the diversity of compound structures, and their pharmacologically active functional groups contribute to the development of new drugs. Therefore, researchers tend to discover novel natural products from the natural world[2]. However, as the boom in natural product discovery gradually subsides, the drawbacks of traditional methods for natural product discovery become apparent: unstable production of biological materials, difficulty in discovering natural products, long and low-yield experimental cycles, and environmental damage.

Simultaneously, in the early 21st century, major pharmaceutical companies began discontinuing drug development plans based on natural products. Due to this series of reasons, the difficulty in exploring novel natural products has significantly increased, leading to a decline in the conversion rate of these novel natural products into new drugs over the years.

Despite the tremendous efforts exerted by researchers involved in natural product isolation, the low abundance of novel natural products often only supports structural identification and the publication of research papers. This limited quantity hinders further activity evaluation and subsequent drug development studies, thus preventing the full exploration of the pharmaceutical potential of these natural products.

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1.2. Synthetic biology facilitates the innovative discovery of natural products

Synthetic biology, as an emerging interdisciplinary field in the 21st century, aims to use biological systems as platforms to make targeted modifications and impart new functions to living organisms. Synthetic biology methods such as heterologous biosynthesis and genome mining provide efficient and convenient new approaches to address the various challenges encountered in the production and discovery of natural products.

1.2.1 Activate silencing gene clusters

Fungi are one of the most important microbial resources for drug discovery due to their ability to produce structurally diverse natural products. It is well known that fungi possess numerous biosynthetic gene clusters (BGCs) encoding various natural product biosynthetic pathways. With the rapid development of genome sequencing technology and bioinformatics tools, a large number of undiscovered BGCs have been predicted. Taking Streptomyces as an example, it is conservatively estimated to be capable of producing 150,000 natural products, yet only 5% have been predicted so far[3].

Due to strict regulation within the organism, some BGCs encoding natural products remain silent in vivo. Thus, activating silent gene clusters provides a strategy for further natural product discovery. Currently, there are two main strategies for activating silent genes: targeted activation of silent BGCs and non-targeted activation of silent BGCs.

Targeted activation of silent BGCs involves knocking in strong promoters upstream of the target BGC to initiate biosynthetic pathways. The research group led by Huimin Zhao at the University of Illinois efficiently and accurately used CRISPR/Cas9 technology to introduce heterologous promoters into the Streptomyces genome to activate BGCs (Figure 2), resulting in the production of new metabolites, including a novel pentangular polyketide in green Streptomyces[4].

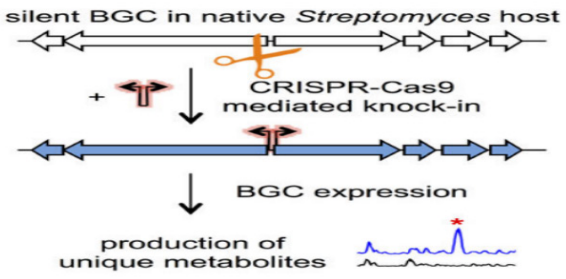

Figure 2. Using CRISPR-Cas9, efficient and precise introduction of promoter cassettes (red arrows) drive expression of biosynthetic genes (blue) and trigger the production of unique metabolites (*) that are not detected for the parent strain. Reference: Same as Reference [4]

Non-targeted activation of silent BGCs does not require clear genomic information to achieve the activation of silent BGCs. Professor M.R. Seyedsayamdost's team has developed a high-throughput elicitor screening method coupled with imaging mass spectrometry (HiTE-IMS), which does not rely on genetic manipulation systems. This method utilizes multiple small molecules as elicitors to induce the activation of silent BGCs in both sequenced and unsequenced bacteria. Finally, imaging mass spectrometry is employed for high-throughput detection of the produced metabolites[5].

1.2.2 Heterologous biosynthesis

Due to the fact that the majority of microorganisms are not cultivable in the laboratory, researchers face challenges in designing and modifying biosynthetic pathways within natural hosts to enhance production efficiency. Consequently, transferring genomes from natural hosts to model organisms with well-established genetic manipulation systems allows for the rapid acquisition of compounds encoded in biosynthesis and the discovery of novel natural products.

By employing synthetic biology methods to systematically expand the chemical space surrounding target natural products, new analogs of natural compounds can be generated. These analogs, not pre-programmed in nature, exhibit higher degrees of modification compared to programmed natural products[6].

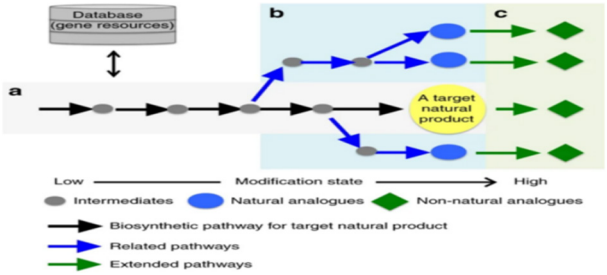

Figure 3. Strategy for expanding the chemical space of a target bioactive natural product via a synthetic biology approach. Reference: Same as Reference [6]

Taking decalin-containing diterpenoid pyrones (DDPs) as an example, a natural product with anti-proliferative effects on cancer cells, Professor Teigo Asai and his team at the Graduate School of Pharmaceutical Sciences, Tohoku University, extended the DDPs synthesis pathway through techniques such as gene mining and heterologous recombination (Figure 3). This effort resulted in the production of 15 new DDPs analogs[6].

Despite limited research on the biosynthesis of DDPs, previous studies estimated biosynthetic gene clusters in entomopathogenic fungi. Building upon this information, Professor Teigo Asai and his team conducted gene mining, designing five natural pathways from biosynthetic gene clusters. These pathways were then reconstructed in Aspergillus oryzae NSAR1, an excellent heterologous host for fungal natural product production, to generate all intermediate and final products.

By extending the pathway through combinatorial biosynthesis, involving the addition of extra modifying enzymes to the natural DDPs pathway, a total of 22

DDPs were produced, including 15 previously unreported analogs (Figure 4). These analogs exhibit diverse biological activities, such as inhibiting cytotoxicity against cancer cell lines by suppressing mitochondrial complex III, anti-proliferative activity against cancer stem cell-like cells, anti-HIV effects, and prevention of amyloid-β (Aβ) aggregation at the nucleation stage, among others.

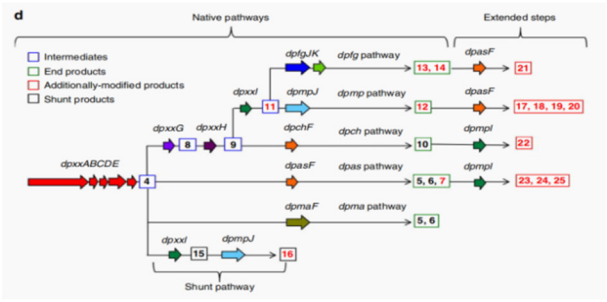

Figure 4. Genome mining and design of DDP pathways. Reference: Same as Reference [6]

2. Validation target

The screening and validation of drug targets are crucial stages in the process of developing new pharmaceuticals, constituting a highly resource-intensive endeavor. Therefore, drug development requires a robust platform for the discovery and validation of drug targets. In the early stages, this platform relied on the model of "gene knockout" in animals. While this technique allowed for the knockout of individual genes on chromosomes, it lacked scalability and the ability to knock out genes vital for embryonic development or those potentially leading to embryonic lethality. Additionally, it faced challenges such as long cycles and high costs, severely hindering the drug development process.

Subsequently, the advent of RNA interference technology (RNAi) significantly shortened the time for screening drug targets and lead compounds. RNAi could effectively and selectively target the mRNA of specific genes, inhibiting the expression of the target genes. However, RNAi technology suffered from issues such as poor reproducibility and serious off-target effects, sometimes leading to entirely erroneous results.

CRISPR/Cas9, with its powerful gene-editing capabilities, is considered one of the most valuable tools for target validation. It has been widely applied in the field of screening and validating drug targets, laying a solid foundation for the development of novel pharmaceuticals.

2.1. The mechanism of action of CRISPR/Cas9 system

The CRISPR/Cas9 system consists of a set of genes comprising the CRISPR array and the Cas protein. CRISPR is an acronym for Clustered Regularly Interspaced Short Palindromic Repeats, and Cas stands for CRISPR-associated protein. The CRISPR/Cas9 system comprises three key components: CRISPR RNA (crRNA), transactivating crRNA (tracrRNA), and the Cas9 endonuclease. A portion of the crRNA sequence can base-pair with tracrRNA, forming a chimeric RNA (tracrRNA/crRNA), while another part can base-pair with the target DNA sequence. This chimeric RNA can recognize specific NGG sequences, guiding the Cas protein complex to cleave the DNA double strand at that specific site[7]. Modification of the chimeric RNA (tracrRNA/crRNA) can lead to the construction of a guiding sgRNA, simplifying the CRISPR/Cas9 system into two components: the Cas9 endonuclease and sgRNA.

When Cas9 cleaves the target DNA, it induces a double-strand break (DSB), triggering the cell's DNA repair mechanism. Non-homologous end joining (NHEJ) repair mechanism can result in random insertions or deletions of multiple bases, causing severe mutations such as frameshifts and missense mutations in the gene encoding a protein, hindering its normal function[8]. On the other hand, homologous recombination (HR) repair mechanism enables precise genetic modifications, such as point mutations, insertions, and epitope tagging [9].Therefore, through these two repair mechanisms, CRISPR/Cas9 technology can achieve both gene knockout and gene knock-in for genome manipulation.

2.2. Application of CRISPR/Cas9 in drug target screening and validation

In the development of novel drugs, the mutual validation between drugs and targets is indispensable. Combining CRISPR/Cas9 technology with whole-genome sequencing and drug resistance mutation screening can efficiently validate targets.

In 2014, Tim Wang's team[10] utilized a library containing 73,000 sgRNA sequences to screen near-haploid human HL60 cells and pseudo-diploid leukemia KBM7 cells. They verified that target genes MSH2, MSH6, MLH1, and PMS2 could repair DNA damage caused by nucleoside analog 6-thioguanine (6-TG), and that the target gene TOP2A could resist the toxicity of etoposide.

In an article published in April 2017, researchers employed macrophages derived from human induced pluripotent stem cells (iPSCs) to study the interaction between macrophages and Chlamydia in vitro[11].Utilizing CRISPR/Cas9 technology, they performed biallelic gene knockout in human iPSCs and, based on RNA sequencing/proteomics analysis, demonstrated the critical roles of the IRF5 gene and IL-10RA gene in resisting Chlamydia infection within the immune system. This discovery identified new drug targets for treating diseases caused by Chlamydia.

Currently, the strategy for screening using CRISPR/Cas9 technology mainly involves inducing mutations in the 5' exons of candidate genes. The drawback of this method is the tendency to generate in-frame mutations retaining partial functionality, which may result in a lack of significant phenotypic differences even in the presence of strong genetic correlations. To overcome this issue, researchers at Cold Spring Harbor

Laboratory [12] targeted CRISPR mutations to exons encoding functional protein domains, leading to the generation of more null mutations and significantly improving the efficiency of negative selection. In this study, they screened 192 chromatin regulatory regions in mouse MLLAF9/NrasG12D acute myeloid leukemia cells, identifying six known drug target genes and 19 potential drug targets. This indicates significant optimization potential for CRISPR/Cas9 technology in screening and validating cancer drug targets.

2.3. CRISPR gene editing identifies mutations that produce drug resistance

The multidrug resistance of bacteria is a problem faced by the global medical industry. Bacterial multidrug resistance refers to bacteria developing resistance to multiple antimicrobial drugs with different mechanisms of action. Most drug-resistant bacteria can resist almost all antibiotics and are referred to as "superbugs." The development of new antibiotics was once the main approach to tackling bacterial resistance, but it faces challenges such as long research and development cycles, high costs, and low success rates, making it difficult to keep pace with the rapid development of bacterial multidrug resistance.

The CRISPR-Cas system, widely present in bacteria, consists of regularly spaced short palindromic repeat sequences and associated proteins and plays an important role in bacterial immune defense. As a novel gene editing tool, the CRISPR-Cas system is gradually being applied in the prevention and control of multidrug-resistant bacteria.

Research has found that horizontal gene transfer (HGT) may facilitate the exchange of genetic material among different bacteria, which is a significant factor in the formation of bacterial multidrug resistance. By integrating sequences encoding the CRISPR-Cas system or its components onto plasmid vectors, researchers can target and cleave drug-resistant genes, thereby reducing the resistance of bacteria to antibiotics.

The CRISPR-Cas9 system was first applied to eliminate drug-resistant genes in resistant bacteria. Researchers have used the CRISPR-Cas system to kill host bacteria carrying drug-resistant genes by targeting them to the chromosome[13], inducing endogenous CRISPR-Cas systems in prokaryotes to remove resistance genes or plasmids[14], increasing the copy number of guide RNA (gRNA) to drive more efficient destruction of high-copy plasmids containing resistance genes and having the potential for more precise editing of target genes[15], inducing Cas9 expression and targeting replication sequences of drug-resistant plasmids to effectively remove plasmids carrying resistance genes[16], and reducing the efficiency of conjugation between plasmids containing resistant genes and recipient bacteria[17] (such as Phnshp45).

These technologies not only have prospects for reducing the resistance of clinical pathogens but also have important applications in genetic manipulation of clinically isolated pathogens and identification of key drug-resistant genes.

3. Scale production

Whether a new drug can be produced on a large scale is a crucial factor in its market entry. Using synthetic biology techniques, it is possible to genetically engineer host cells such as Escherichia coli and fungi, followed by cultivation in large-scale bioreactors. Currently, synthetic biology has made significant breakthroughs in the biosynthesis of terpenoid compounds derived from plant sources, including active medicinal ingredients like artemisinin, paclitaxel, lycopene, and others.

3.1. Chassis cell construction

Currently, the construction of microbial chassis cells roughly falls into two strategies that can be used independently or complementarily: "top-down genome streamlining" and "bottom-up genome synthesis."

Genome streamlining refers to the extensive deletion of non-essential coding and non-coding regions from the genome, resulting in a "minimal genome." Before streamlining the genome, preliminary identification of essential and non-essential genes can be achieved through model analysis using bioinformatics tools and comparing existing databases of essential and non-essential genes[18]. Technologies such as RNAi and CRISPR are often employed to trim non-essential genes, determining essential from non-essential genes.

The top-down genome streamlining approach is relatively straightforward, but it requires a profound understanding of genome composition and function. However, it also has limitations such as being time-consuming and costly.

Genome synthesis is a bottom-up forward engineering strategy, starting from biological components to modules and then to genome synthesis assembly and chassis cell construction. During the genome synthesis process, systems biology techniques play a crucial role in the characterization and assessment of synthesized genome performance. A milestone achievement in synthetic biology history was the synthesis of the minimal artificial genome by the Craig Venter team in 2016, consisting of 531 kb with 473 genes[19]. This marked the artificial synthesis of a prokaryotic genome. Successful examples of genome synthesis in eukaryotes include the construction of a single-chromosome synthetic yeast, which has made significant breakthroughs in the Synthetic Yeast 2.0 Project with a 12 Mb whole-genome synthesis plan[20].

3.2. Large-scale production of natural medicine

Using artemisinin production as an example, malaria remains a global health concern, threatening the lives of 300-500 million people. Artemisinin, a sesquiterpene lactone peroxide extracted from Artemisia annua, exhibits potent anti-malarial properties. However, its scarcity renders it unaffordable for most malaria patients.

Currently, employing synthetic biology methods to semi-synthesize artemisinin or its derivatives presents a potentially cost-effective, environmentally friendly, high-quality, and reliable source of artemisinin. Researchers have engineered yeast strains to produce artemisinic acid through a three-step process[21]. This involves enhancing farnesyl pyrophosphate (FPP) biosynthesis pathway engineering to increase FPP production and expressing amorphadiene synthase to create yeast strains capable of producing high levels of artemisinic acid, making microbial production a viable source within this potent anti-malarial drug family.

A conservative analysis suggests that this approach could significantly undercut its current price, saving costs. Moreover, this bioprocess should not be susceptible to factors that might affect plant cultivation, such as weather or political climates. Additionally, environmentally friendly processes can extract artemisinic acid from microbial sources without concerns about contamination from other terpene compounds produced by plants, thus increasing the complexity of artemisinin production while reducing purification costs.

4. Specific application of synthetic biology in the field of drug research and development

4.1. Paclitaxel

Paclitaxel is a diterpenoid compound with anticancer activity, extracted from the Taxus genus of plants. Its mechanism of action involves promoting microtubule polymerization to inhibit the mitosis of tumor cells, making it one of the most outstanding natural antitumor drugs in the pharmaceutical market.

In 2019, researchers from the Chinese Academy of Sciences achieved the heterologous synthesis of the key intermediate, taxadiene, of paclitaxel in Nicotiana benthamiana, a plant-based platform (Figure 5)[22]. The researchers designed a partial pathway for paclitaxel by introducing taxadiene synthase (TS), taxadiene 5α-hydroxylase (T5H), and cytochrome P450 reductase (CPR) into the chloroplast of N. benthamiana. By combining this compartmentalization strategy with enhanced precursor supply and transfer of the taxadiene biosynthesis pathway, a higher accumulation of taxadiene was achieved, resulting in a yield of 56 μg/g.

Compared to traditional microbial and yeast platforms, plant-based platforms are more compatible with heterologous expression of plant enzymes such as cytochrome P450, owing to suitable intracellular systems, the availability of cofactors, and protein folding mechanisms. This work in high-biomass plants establishes a starting point for optimizing paclitaxel production in heterologous plants in the future.

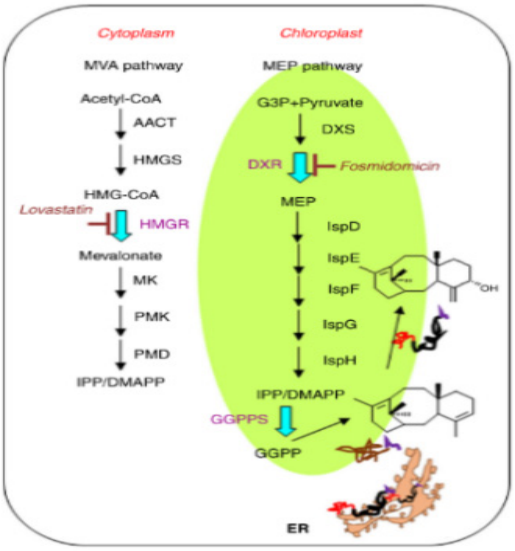

Figure 5. the synthesis of paclitaxel intermediates in cells. Reference: Same as Reference [22]

4.2. Vincristine

Vincristine belongs to the monoterpenoid alkaloid class, which has strong biological activity. Since the anticancer drug Changchun alkaloid was introduced in 1958, it remains an active drug on the clinical front line.

In 2022, researchers from the Chinese Academy of Sciences collaborated with foreign institutions to engineer the yeast chassis, resulting in the production of two precursor compounds, Wendoling and Changchunine. These two precursors can ultimately form a new derivative of Vincristine[23].The research team performed a total of 56 gene edits on the yeast strain, integrating 31 biosynthetic steps into the yeast. This study represents the longest biosynthetic pathway ever reprogrammed in microorganisms.

In 2023, the teams led by Jay D. Keasling and Jia-Chang Lian respectively achieved the first total synthesis of Vincristine precursor using the model organism Saccharomyces cerevisiae and the non-model organism Pichia pastoris[24]. The Lian team divided the synthesis pathway of Changchunine into three functional modules, with Isochisopeptide and Jinggaiol as the key junctions.

By modifying the crucial protein CrPAS, increasing the copy number of rate-limiting enzyme genes, and engineering the chassis cells, they carried out fed-batch fermentation in a 1 L bioreactor, achieving a yield of 2.57 mg/L.

4.3. Tropane alkaloids

Tropane alkaloids, belonging to the tropane alkaloid class, is a fundamental clinical drug. However, the low natural abundance of tropane alkaloids in plants leads to their high cost.

In 2020, Christina D. Smolke's research group from Stanford University achieved the first artificial synthesis of tropane alkaloids in yeast[25]. The team designed a biosynthetic pathway in the yeast chassis, consisting of five functional modules (Figure 6). Modules I/II and III enable the de novo biosynthesis of acyl acceptor and donor components. Module IV allows the modification of the tropaneamine (TA) scaffolds to produce hydroxyhyoscyamine and hyoscyamine. Module V involves the central acyl transferase reaction, connecting the upstream acyl acceptor/donor biosynthesis with downstream scaffold modification.

Using functional genomics approaches, the researchers discovered a key reductase enzyme HDH that converts tropinone aldehyde to tropine, as well as successfully screened LS enzymes capable of experimental transmembrane expression. Finally, the entire synthetic pathway was applied to the yeast chassis cells, and with fermentation optimization, a yield of 60 mg/L was achieved.

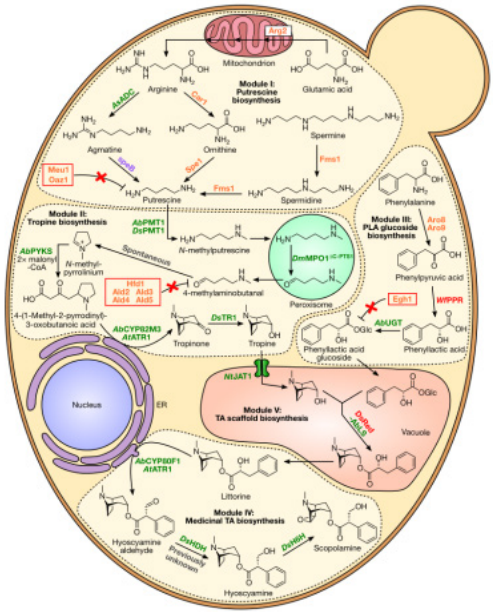

Figure 6. Modular pathway construction for scopolamine biosynthesis in yeast. Reference: Same as Reference [25]

5. Summary and prospect

With the development of society and the increasing demand for health and nutrition, there are higher expectations for the large-scale industrial production of drugs.

Compared to traditional methods such as plant extraction and chemical synthesis, synthetic biology techniques offer advantages in constructing artificial cell factories that are unaffected by external environmental conditions, have fast growth rates, and can be regulated. The development of synthetic biology has made it possible to produce valuable natural products, overcoming the limitations of traditional extraction methods that cannot be commercially scaled. With the rapid advancement of biological informatics and DNA gene sequencing technology, industrial production techniques for natural products in our country continue to improve.

However, despite the vast prospects for the development of synthetic biology in the field of therapeutics, it is important to acknowledge that challenges still exist.

Firstly, standardizing genetic elements is difficult, and they cannot be simply "plug and play" in different cell systems. Due to a limited understanding of how to simulate a host organism, the situation becomes more complex. Therefore, the focus should be on determining the minimal gene set required for cellular activity, enabling the construction of cells from the bottom up.

Secondly, the introduction of genetic circuits into cells may not be permanent or stable due to environmental influences. This could result in differences between batches, potentially affecting drug evaluation and production.

Thirdly, in addition to the coding genome, there are non-coding elements that also play a role in regulating gene expression. However, further research is needed to understand this process.

Finally, unlike engineering systems where components can be easily modified and transferred, biological components are less transferable between host organisms due to their lack of orthogonality, which can interact with the host's genes, proteins, and metabolites.

In conclusion, while synthetic biology has great potential for the industrial production of drugs, there are still challenges that need to be addressed. Ongoing research and development efforts can help overcome these challenges and further advance the field of synthetic biology.

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