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Engineering in complex systems

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The implementation of the engineering design cycle of measure, model, manipulate would drastically enhance the success rate of biotechnological designs. Recent progress for the three elements suggests that the scope of the traditional engineering paradigm in biotechnology is expanding. Substantial advances were made in dynamic *in vivo* analysis of metabolism, which is essential for the accurate prediction of metabolic pathway behavior. Novel methods that require variable degrees of system knowledge facilitate metabolic system manipulation. The combinatorial testing of pre-characterized parts is particularly promising, because it can profit from automation and limits the search space. Finally, conceptual advances in orthogonalizing cells should enhance the reliability of engineering designs in the future. Coupled to improved *in silico* models of metabolism, these advances should allow a more rational design of metabolic systems.

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Introduction

Biological systems are equipped with an impressive number of enzymes, enabling a myriad of catalytic reactions which can produce diverse chemical compounds. This amazing capability is established by a complex cellular apparatus that records and integrates environmental signals. This apparatus directs the production of enzymes as parts of efficient pathways in the right amount at the right time. Attempts to obtain strains with an improved production of the target compound relied first on (now) classical strain development programs of iterative cycles of mutagenesis, screening, and testing on larger scale. These methods were later complemented by increasingly rational methods of strain modification. However, the construction of effective overproducing strains requires substantial modification of the productions strain's genome. This was first suggested by the unifying concepts of mass balancing, stoichiometry, and mathematical modeling and reinforced later with the advent of molecular system-wide analysis tools.

The development of rather cheap *de novo* DNA synthesis, the current scope of which has recently been demonstrated [1••], now provides a technology to match the requirement of system-wide strain modifications. Unfortunately, there exists hardly any conceptual framework to use this efficiently. The traditional human strategy to translate scientific insight into technical innovation is engineering. In contrast, the most successful strategy in biotechnology so far has been evolution. We argue that the potential scope of construction that comes with large scale *de novo* DNA synthesis reinforces the question for the most suitable strategy of constructing efficient biotechnological systems.

Metabolic engineering provides one of the most advanced rational design frameworks in biotechnology, and should therefore take particular advantage of the novel technological capabilities. It will be interesting to follow which strategies will be adopted by metabolic engineering to guide its future strain improvement efforts. In other words: will we use the capacity of writing novel DNA code to design or to evolve cells in the future?

Pathway engineering: the engineering design cycle

The hallmark of design is the engineering design cycle with its three elements of 'measure, model, manipulate'. The properties of a designed system are measured, compared with the simulations from a sufficiently detailed model, and after adaptation the model is used to instruct the design of the next version of the system. The system is then assembled from properly characterized parts. Importantly, systems and parts are designed in such a way that the parts can be implemented without affecting other system components. In other words, all design relevant information can be ascribed to the parts only. This system design property is known as orthogonality from computer science. By iteratively going through the design cycle for a limited number of times, even large functional systems can be constructed effectively with limited effort. Clearly, living cells do not comply with these specifications: they are neither orthogonal nor sufficiently understood. They grow, react, and evolve — the system changes its composition during growth and in response to the environment, and its underlying genetic code changes over time. Therefore, the justified question is: how far can engineering actually reach? We will explore in the following recent advances

along the engineering cycle with metabolic engineering as an example and then ponder the question whether a living system can be adapted to the requirements of classical systems engineering.

The engineering design cycle: modeling

The goal of modeling is to find a mathematical description of biological systems behavior, which can then be used to predict the behavior of the system *in silico* rather than doing experiments. A large number of different models addressing the various aspects of biological systems are currently available, the majority of which addresses the topology of biochemical networks. Topology refers here to the composition of a network in terms of absence or presence. Genome scale metabolic models have expanded rapidly within the last decade and are available for more than 50 organisms. Genome scale metabolic models directly link metabolites to genes, currently still neglecting transcription, translation and biochemical reaction dynamics. However, the increasing complexity and accuracy of these models enables a near comprehensive *in silico* prediction of phenotypes (reviewed in [2]). Furthermore, genome scale topological models or subsets of them are the underlying reference for ^{13}C-based metabolic flux analysis, which allows the determination of a steady-state flux distribution through a reaction network [3].

On the topology level, biochemical *de novo* pathway design tools have been established to perform retrobiosynthesis exploiting either existing [4–6] or even hypothetical pathways [7•]. These models explore the space of existing or hypothetical reactions to connect the target molecule to the starting chemical, but usually neglect how the pathway is embedded into the overall biochemical reaction network of the corresponding host organism, its dynamics, and its thermodynamics. Recently, a more comprehensive approach was used to theoretically analyze opportunities for improved biological CO_2-fixation. It integrated kinetic enzyme data from the literature for at least one key step [8•].

However, the introduction of kinetic data is essential for rational engineering. The flux through an enzyme is not only a function of its presence, but of enzyme and metabolite concentrations and the intrinsic kinetic properties of the enzyme: for example, a typical enzyme network evolved under the cellular requirement of homeostasis is able to easily compensate single interventions into the network [9] and thus frustrate simple engineering strategies. Therefore, one crucial challenge is to build a conceptual framework to combine kinetic information with stoichiometric models. However, current efforts still suffer from inadequate information on the dynamic behavior of the various system members [10–12]. Consequently, dynamic models focusing on a subset of metabolic reactions have been proposed [13–15], which however still suffer from poor identifiability.

Measuring

Clearly, the development of better dynamic models is also a function of the available experimental technology to analyze system dynamics. Here, quantitative metabolomics, which determines absolute concentrations of intracellular metabolites, is a crucial advance. Different platforms are currently available for analyzing metabolite concentrations but LC–MS appears to be the most comprehensive method for metabolite quantification in a single run [16] and can be used for quantification of more than 100 intracellular metabolites in *Escherichia coli* [17]. However, the high turnover rates and the intracellular nature of the majority of metabolites require very rapid and reproducible quenching and metabolite extraction procedures [18]. Once this is established, two strategies can be followed to extract *in vivo* kinetic data: first, the dynamics after perturbation with a non-labeled substrate can be used to elucidate kinetic data and points of flux control [19,20]. Alternatively, the substrate is changed to partially ^{13}C-labeled followed by tracking the change of the labeled/non-labeled concentration ratio over time. This can be used for model-based analysis of intracellular fluxes and determinations of turn-over times of metabolite pools under industrially relevant, non-steady-state conditions. It is also expected to increase the accuracy of kinetic parameter estimations in dose–response experiments [21].

Another limitation in dynamic metabolomic measurements is the requirement of limited observation times to prevent cellular adaptation. Recent experiments demonstrated the measurement of the intracellular concentration of single proteins and applied this to the analysis of protein dynamics in yeast [22••]. Furthermore, a high throughput method was developed to adapt this quantitative proteomic analysis to other organisms [23], suggesting that this particular limitation might be overcome in the future.

Although these methods confirm substantial progress in the elucidation of system dynamics, they still require extensive sample preparation and handling procedures. Additionally, the limited set of perturbations that is currently possible (limited number of carbon sources, salts and gases plus genetic manipulation of specific members of the network with unpredicted consequences on the overall composition of the network) intrinsically limits the information that can be gathered. In summary, the implementation of dynamic metabolomic measurements is advancing, but does not yet meet the requirements for parameterization of reliable mechanistic kinetic models.

Manipulation

The well-established steady-state modeling and measurement tools allow very useful predictions on pathway

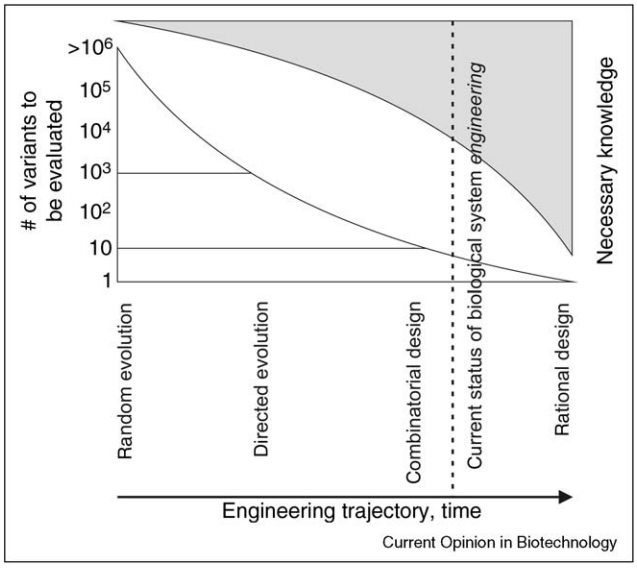
optimization, such as reaction network topology and yield increases. They also allow generating hypotheses about crucial factors for pathway performance. However, optimal pathway design has to consider that enzymes in a pathway act in concert. The control over pathway flux can be distributed over several enzymes in (and out of) the pathway. This means that simple overexpression of a recombinant gene may have severe adverse effects because a specific intermediate might accumulate or decrease in concentration beyond a critical value. Correspondingly, fine tuning of reaction network dynamics by modifying gene expression of pathway members has an enormous optimization potential [24]. This can be achieved in a directed evolution approach: cell-wide gene expression tuning on the transcriptional level has been performed for example, through modulating promoter strength by sigma-factor engineering [25]. Alternatively, tunable intergenic regions [27•] or random replacement of ribosome-binding sites [26••] can be used to control mRNA translation. All three approaches are based on random mutagenesis of the respective genetic element followed by high throughput screening for the desired phenotype. Consequently, the only decision to take is which element to engineer and then the best phenotype is derived by directed evolution (Figure 1). As long as rapid screening protocols can be developed, this is a feasible strategy.

One obstacle for implementing such focused directed evolution strategies is the limited number of different chromosomal sites that can be accessed simultaneously. This problem has been comprehensively addressed by 'multiplex automated genome engineering' (MAGE) [26••]. Degenerate DNA oligonucleotides were used to transform for example, *E. coli* and to site-specifically modify chromosomal sites with the help of the lambda Red beta function. By automating the process of growth, electroporation, recovery and return to growth, it is possible to carry out multiple rounds of genome adaptation on many different sites per day. The potential of the method was shown by providing oligonucleotides for engineering 24 genomic targets including ribosome binding sites of 20 genes. Going through 35 cycles in 3 days produced a strain which synthesized five times more lycopene than the reference strain.

The search space to be explored at a specific DNA-sequence site can be further constrained in two ways: first, computational design tools can be used, for example to rationally engineer ribosome-binding sites. Here, a novel method is based on calculating total Gibbs free energies of ribosome binding and translation initiation. This allows the calculation of a relative translation rate or the prediction of a ribosome binding sequence with a particular relative translation rate [27•]. The approach can reduce the screening of thousands of promoters or ribosome-binding sites, but still requires the analysis of several variants to obtain one with the desired properties. Similar tools become available for promoter prediction [28]. Alternatively, a limited number of well-characterized and pre-manufactured sets of expression tools can be used, such as promoter variants of the same type but of different strengths [29]. On pathway level multiple expression signals need to be coordinated to obtain a well-tuned system. This can result in large numbers of combinations to be tested and might be called 'combinatorial design' (Figure 1). The advantage of combinatorial design is that the elements that are recombined are well characterized. Additionally, analyzing several suboptimal designs generate straightforward hypotheses for further improvement of the pathway performance. Unfortunately, combinatorial design is currently limited by the context-sensitivity of many expression signals, which frequently increases the combinatorial search space.

Next, the assembly of proper parts into larger systems has to be addressed. Already the straightforward assembly of a pathway of 10 kbp (e.g. from 25 separate parts such as genes, ribosome-binding sites, regulators, promoters, and terminators) is not trivial, even when using advanced versions of standard cloning protocols [30]. However, *in vivo* recombination in yeast can be used to efficiently assemble multiple, overlapping DNA pieces from oligonucleotides to 50 kbp linear fragments [31,32]. Remarkably, the efficiency of *in vivo* homologous recombination can be paralleled *in vitro*. Extended single strand ends are used for hybridization of neighboring fragments followed by repair of gaps and ligation. This way, up to 900 kbp of

Figure 1



The relationship between required knowledge and different manipulation strategies on the road to engineering design.

DNA have been assembled *in vitro* and *E. coli* has been transformed with DNA fragments of up to 300 kbp [33•].

In principle, these assembly protocols should also be suitable for the directed evolution and combinatorial design approaches mentioned above. It should be possible to incorporate specific diversified sections, as long as these sections have overlapping ends for subsequent integration. The combinatorial design strategy allows in principle a substantial reduction of the search space, while the directed evolution approaches are only feasible if a suitable screening or selection method is available. At the same time combinatorial design requires a more comprehensive knowledge on the system (Figure 1). The combinatorial design method is 'recursive' in character — an existing set of expression elements is assembled into a pathway and later specific elements are exchanged for others. Such recursive schemes are particularly well suited for the automation of assembly, as they permanently return to the same pool of DNA molecules along the same protocol [34•].

Addressing fundamental limitations of the design cycle

The current limitations for engineering complex biological systems are substantial gaps in the understanding of the complex cellular processes. This is reflected in the context-sensitivity of the building blocks used to construct metabolic systems. The complexity is a derivative of evolution and guarantees robust biological network operation [35], but it reduces the engineering success rate. One might therefore argue that reducing the complexity of biological systems will facilitate its engineering — a hypothesis that still needs to be confirmed in the laboratory. Two strategies try to address cellular complexity: reduction of system size and insulation between subsystems. The former approach is accounted for by the various efforts of genome reduction or even defining a minimal genome that would still allow independent growth under specified laboratory conditions [36]. A technological breakthrough for the minimal genome project is the chemical synthesis and transplantation of a Mycoplasma genome [1••]. Conceptually, a tested experimental procedure is now available starting from hypotheses on genome composition via non-template-driven DNA-code generation to testing the construct in a cytoplasm.

For pathway insulation, one prime example is the development of an orthogonal system for the insertion of non-natural amino acids into recombinant proteins in *E. coli* cells [37,38••]. In contrast to previous attempts which suffered from cross-reactivities, the recently implemented system limits the integration of unnatural amino acids into the specific protein of interest. This is ensured by artificially evolved orthogonal ribosomes, which only translate the mRNA molecules encoding for the protein of interest using artificially evolved tRNAs that can only interact with the orthogonal ribosomes. This way, cross reactions are effectively excluded and even a novel genetic code can be implemented in a chemically insulated cellular subsystem. It is worth emphasizing that the underlying experimental methods for implementing such a quasi-orthogonal system were combinations of positive and negative selections — in other words, evolution.

A more subtle form of insulation is the channeling of intermediates between co-localized enzyme activities. Co-localization accelerates metabolite conversion by increasing the effective metabolite concentration in the proximity of the converting enzyme. Correspondingly, this limits the impact of the metabolite elsewhere because metabolite concentrations can be lower. This principle has been tested with a recombinant mevalonate synthesis pathway in *E. coli*. The flux through this pathway could be substantially enhanced by tethering the three involved proteins to each other in a specific stoichiometry [39••]. A similar success was reported for the production of glucaric acid via a three-enzyme pathway [40].

Conclusion

The examples discussed above indicate the various efforts for expanding the engineering basis of biological systems. In particular the development of modeling and analytical techniques provide a mechanistic formulation of complex intracellular behavior. Success in this area would foster a more comprehensive adoption of the engineering design principles, but is currently hampered by many experimental hurdles. At the same time, complexity in biological engineering is only very rudimentarily dealt with, although essential technological foundations have been laid and conceptual proof-of-principles delivered.

The duality between engineering design and evolutionary approaches is most obvious for the 'manipulation' sector of the engineering cycle. Here, strain construction relies on a broad range of methods which employ a variable degree of engineering principles. Random evolution based on mutagenesis and brute force screening only requires limited knowledge on the system but the cause for the beneficial effect frequently remains unclear. Directed evolution needs at least knowledge on the element that should be modified (e.g. gene, promoter, or ribosome binding site), while a detailed optimization strategy is not needed. Combinatorial design follows a semi-rational strategy based on characterized parts and compensates for the current lack of detailed instructions for a comprehensive blueprint for optimization (Figure 1).

Nevertheless, more and more similarities to 'traditional' engineering disciplines become clearly visible in biotech-

nology and specifically metabolic engineering ranging from pre-manufactured parts to ideas such as orthogonality. More of these efforts will be needed to increase the reliability in terms of outcome to levels that are comparable to classical engineering disciplines. However, some conceptionally new approaches are emerging and the first successes are encouraging.

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