*Graphical abstract (for review)

Design

Learn

Build

Test

Complex Systems in Metabolic Engineering

James D. Winkler^a, Keesha Erickson^{a,2}, Alaksh Choudhury^{a,2}, Andrea L. Halweg-Edwards^a, Ryan T. Gill^{a,1}

^a*Department of Chemical and Biological Engineering, University of Colorado-Boulder, Jennie Smoly Caruthers Biotechnology Building, Research Park, Boulder, CO USA 80303*

Abstract

1 Metabolic engineers manipulate intricate biological networks to build efficient bio-
2 logical machines. The inherent complexity of this task, derived from the extensive
3 and often unknown interconnectivity between and within these networks, often pre-
4 vents researchers from achieving desired performance. Other fields have developed
5 methods to tackle the issue of complexity for their unique subset of engineering prob-
6 lems, but to date, there has not been extensive and comprehensive examination of
7 how metabolic engineers use existing tools to ameliorate this effect on their own re-
8 search projects. In this review, we examine how complexity affects engineering at
9 the protein, pathway, and genome levels within an organism, and the tools for han-
10 dling these issues to achieve high-performing strain designs. Quantitative complexity
11 metrics and their applications to metabolic engineering versus traditional engineer-
12 ing fields are also discussed. We conclude by predicting how metabolic engineering
13 practices may advance in light of an explicit consideration of design complexity.

Email addresses: james.winkler@colorado.edu (James D. Winkler),
keesha.erickson@colorado.edu (Keesha Erickson), alaksh.choudhury@colorado.edu (Alaksh
Choudhury), andrea.edwards@colorado.edu (Andrea L. Halweg-Edwards)

¹Corresponding author. Tel: (303) 492-2627, email: rtg@colorado.edu.

²These authors contributed equally to the manuscript.

Introduction

14 Metabolic engineering has undergone profound changes from its initial origins in
15 bioprospecting [1], random mutagenesis, and screening. Today, our increasingly large
16 body of knowledge and powerful tools have enabled the creation of a vast array of
17 microbial cell factories [2]. Capabilities continue to accelerate due to advances in un-
18 derlying technologies: automated genome engineering [3], high-throughput genome
19 modification [4], and recombineering [5]. As such, future approaches like whole
20 genome synthesis [6] will provide new levels of control over strain performance. How-
21 ever, similarly rapid improvements in our ability to comprehend what constitutes a
22 successful design that responds to environmental cues effectively and produces the
23 desired performance are lacking [7]. The majority of metabolic engineers still rely
24 on human inference when generating new strains (Figure 1), which typically entails
25 manual examination of metabolic pathways and designs drawn from the personal ex-
26 perience of the experimenter, rather than a defined design algorithm. This approach
27 is not feasible for future large-scale strain construction efforts based on continued
28 advances in DNA synthesis, which will enable the creation of tens of thousands of
29 made-to-order strains modified at the genome-scale. Rather, new forward engineer-
30 ing approaches that automate strain design, construction, and testing while invoking
31 machine learning and related computational techniques are required.

32 The core challenge to the development of such approaches is the fundamental
33 lack of understanding of the rules governing the design of complex biological sys-
34 tems. While there have been significant developments in computational strain design
35 methodology, we still are unable to accurately predict design performance at the de-

gree of accuracy required, as has been noted recently [8]. Creating and manipulating complex systems, however, is a fundamental aspect of almost all engineering fields. For example, as software systems grew beyond a size comprehensible by a single person, researchers began to create formal methodologies and analysis tools to aid in complexity abstraction, enabling productivity gains to continue apace. Most non-trivial software packages today are developed by large teams, where no one individual has the responsibility for (or perhaps knowledge of) the entire codebase. Analogous to the plethora of integrated software development environments, analysis packages, and libraries available to software engineers, similar support tools are under active development by metabolic engineers to improve our ability to cope with the inherent complexity of living organisms and the many unknown variables influencing their behavior.

Given the importance of complexity in the context of metabolic engineering, this review aims to examine several aspects of systems complexity that metabolic engineers commonly grapple with during the strain development process, along with possible ways of reducing its impact on project development. We focus on how complexity impacts three distinct areas that engineers commonly manipulate to improve chemical production: protein engineering, improving or altering enzymes to manipulate an individual facet of metabolism; pathway construction, channeling flux into the desired branches of the metabolic network; and genome engineering, ensuring that all of these modifications interoperate efficiently. Finally, the quantification of complexity and its implications for the practice and scale of metabolic engineering are examined.

59 Protein-level Engineering

60 Much like individual methods are the building blocks for software, the first level
61 metabolic engineers can target for strain improvement are the enzymes responsible
62 for substrate conversion. Design parameters at this level include protein expres-
63 sion, activity, stability, target specificity, and a range of others characteristics [9].
64 Several methods with different knowledge requirements (i.e. complexity) have been
65 developed to assist in this process (Figure 2). Various types of transcription and
66 translation optimization exist, depending on the needs of the experimenter, such
67 as controlling plasmid copy number [10], codon usage [11], promoter strength [12],
68 mRNA stability, and RBS efficacy [13]. These parameters can be changed using a
69 reverse-engineering approach that requires little knowledge of the desired expression
70 goal, or cellular models can be applied to estimate the optimal expression level for
71 the protein of interest. These methods represent a trade-off between experimental ef-
72 fort via knowledge-based approaches, with the former representing a low complexity
73 (in terms of data required) tactic but necessitating reverse engineering versus high
74 complexity model driven analyses that require substantial empirical testing, com-
75 putational development and validation, but can provide physical or statistical bases
76 for engineering suggestions. This classification can be extended beyond expression
77 optimization to sequence-level alterations in the protein itself.

78 The contrast between high complexity data-driven and low-complexity random
79 approaches extends to improving protein function by mutation. Reverse engineering
80 approaches, like error-prone PCR and DNA shuffling, have proven to be highly ef-
81 fective in improving properties such as activity, substrate range, and stability when

82 structural or functional information is unavailable [14]. These techniques have the
83 added advantage of low up-front cost and knowledge required for generating and
84 screening target libraries. As with other library-based approaches, mutagenesis
85 methods can be limited by complex fitness landscapes [15] that arise from epistatic
86 interactions between residues, the screening effort required for large libraries, and
87 subsequent investigation into mechanism improving function. These limitations are
88 inherent to all random approaches, as it is often difficult to understand how phe-
89 notypes are improved through mutagenesis without a conceptual structure-function
90 model. Random methods are, as a result, of generally low conceptual complexity,
91 but their explanatory power can be limited without extensive reconstruction and
92 physical modeling.

93 In contrast to random mutagenesis, forward approaches to protein engineering
94 are able to reduce the experimental effort by identifying residues of interest at the
95 cost of substantially increased algorithmic complexity. A combination of structural
96 analysis, bioinformatics and *de novo* protein design tools have been quite success-
97 ful in developing improved biocatalysts [16]. Structure-activity methods such as
98 proSAR [17] require previously generated data to identify key residues that affect
99 protein function, although the model in this case is purely statistical and lacks the
100 complexity inherent in physical protein models. For proteins with known structures,
101 tools such as Rosetta [18] and SCHEMA [19] reduce library size (trading algorithmic
102 complexity to reduce experimental burden) by eliminating unstable mutants for
103 site saturation mutagenesis and site directed chimeragenesis, respectively. Such a
104 combination has proven effective in metabolic engineering designs [20–23]. The key

105 unifying factor behind all these tools is a reduction in experimental effort relative
106 to random approaches. Despite the high initial cost of developing high complexity
107 methods, these tools can significantly reduce the time, effort, and labor associated
108 with classical protein engineering tools.

109 **Pathway-level Engineering**

110 The same reverse versus forward engineering complexity trade-off also applies
111 at the pathway level. Complexity increases when engineering a pathway, as the
112 number of engineering parameters expands to include interactions among various
113 pathway enzymes and overall cellular metabolism (Figure 3). The key design param-
114 eter metabolic engineers must contend with is gene selection and a number of in silico
115 tools have been developed to assist researchers in selecting the best gene(s) for their
116 pathway based on thermodynamics, pathway length, and other criteria (see [24] for
117 an extensive review). The Biochemical Network Integrated Computational Explorer
118 (BNICE) is one of the most well-known examples of these tools [25]. Additional
119 enzyme selection tools include Metabolic Tinker [26], FMM [27], and Desharky [28].
120 For retrosynthetic approaches, the user selects an end product and pathways to that
121 product are suggested [29–31]. Future tools may be able to predict whether enzymes
122 can operate on non-natural substrates along with accounting for metabolite toxicity
123 issues as well, reducing the difficulty of pathway design further. No matter the under-
124 lying implementation or selection criteria, these tools all allow researchers to abstract
125 the complexity of enzyme selection by employing various modeling techniques.

126 Once the pathway has been specified, it must be optimized for maximum per-

127 formance. The increased number of design parameters at this stage, including the
128 relative expression levels, activities, environmental tolerance, and other properties,
129 represent a significant increase in complexity compared to the engineering of a single
130 protein. Both reverse and forward engineering techniques can be applied to grapple
131 with this problem; examples for the former include high-throughput library gener-
132 ation and screening (or selection) [32–34], global regulatory engineering [35, 36] or
133 combinatorial module optimization [37]. Reverse engineering is necessary in par-
134 ticular when gaps in knowledge present additional complexity; for instance, with
135 limited knowledge, gene expression studies can deliver insight into pathway regula-
136 tion [38, 39], while metabolomics and meta-metabolomics profiling can lead to the
137 discovery of novel biological products [40]. Reverse engineering approaches rely on
138 generation and screening of many potential production strains in order to identify
139 optimal parameter sets post hoc, thereby exchanging screening effort for up-front
140 system modeling. Forward engineering is exemplified by the many computational
141 pathway and metabolic models that guide metabolic interventions [41–43], or those
142 that exploit thermodynamic modeling to create irreversibilities in pathway flux [44].
143 The distinguishing factor between forward and reverse pathway engineering is that
144 forward methods attempt to model sufficient parameters to predict favorable path-
145 way interventions, and require a substantial knowledge base concerning both the
146 organism and enzymes in question.

147 **Genome-level Engineering**

148   Engineering an entire organism poses challenges beyond those commonly encoun-
149   tered at the protein or pathway levels, as described in Figure 4. The number of design
150   parameters increases significantly, as was the case from the protein to pathway tran-
151   sition: redox balance must be maintained [45], flux must be maximized through com-
152   mon metabolic checkpoints [46], and products must be sufficiently exported out of the
153   cell to minimize toxicity [47], among many other parameters. Forward engineering of
154   desirable phenotypes becomes more challenging as a result, and reverse engineering
155   is often used to improve industrially relevant phenotypes due a lack of knowledge
156   concerning the underlying molecular mechanisms for a set of desirable traits. In the
157   absence of a known genotype-phenotype connection, non-targeted methods such as
158   adaptive evolution, genome-scale libraries, and other tools can effectively improve
159   industrially relevant phenotypes [4, 48, 49], including production of specific metabo-
160   lites [50]. Other reverse engineering methods can be applied to individual proteins,
161   such as product-export pumps, to avoid toxicity and increase thermodynamic driv-
162   ing forces on production [51]. Designing self-regulating strains can also ameliorate
163   complexity, as there is no need to manually regulate pathway expression without
164   external inducers to both minimize toxicity and maximize productivity [36]. These
165   approaches reduce the knowledge required for achieving the desired production phe-
166   notype, thereby reducing the complexity of genome-scale engineering compared to
167   forward approaches, such as metabolic modeling.

168   True forward design of an entire genome, in contrast to reverse engineered al-
169   terations, is currently beyond the capabilities of metabolic engineers. Gibson *et al.*

170 took the first steps towards this goal by constructing the first chemically synthesized
171 bacterial genome [6]. Dymond *et al.* have undertaken a related effort to replace yeast
172 chromosome with redesigned analogs as well [52]. However, rational approaches to
173 engineering organisms at this scale will require dramatic reductions in DNA synthesis
174 and sequencing costs, as well as an improved understanding of the minimal sequence
175 requirements for engineering a chassis genome. These efforts will simultaneously
176 rely on and improve whole cell models due to their generation of large genotype-
177 phenotype datasets. The powerful whole-cell model of *Mycobacterium* by Karr *et*
178 *al.* [53] and the original E-CELL model [54] demonstrate some of the physiological
179 insights, including estimates of protein-DNA binding frequency, that can be gleaned
180 from this approach. However, due to the much larger genome size of most common
181 metabolic engineering platform strains, developing realistic and computable whole
182 cell models for these organisms poses additional challenges. Both genome redesign
183 and cell modeling efforts represent the forefront in metabolic engineering complexity,
184 as interactions at all three levels discussed here must be explored in order to build
185 made-to-order biocatalysts.

186 Although the field of genome design is in its infancy at the moment, we expect
187 that future developments in DNA synthesis, cloning, and automated re-engineering
188 of genome architectures will lead to the gradual introduction of dramatically rewired
189 organisms that should exhibit reduced complexity-related issues. However, since
190 even simplistic organisms require sophisticated regulatory and metabolic networks
191 for growth and efficient catalysis, grappling with complexity through a combination
192 of experimental validation, simulation, and modularization will remain a challenge

193 at the forefront of metabolic engineering at all of the scales explored here.

194 **Measuring Complexity**

195 It will soon be possible to generate a comprehensive library of genome-scale de-
196 signs in a single experiment, rather the relatively small-scale projects that currently
197 characterize most metabolic engineering efforts. Taking advantage of this explo-
198 sion in the number of testable designs is extremely challenging, as the subsequent
199 experimental design evaluation and analysis steps remain comparatively slow [55].
200 Complexity assessment can be used to address important aspects of this issue, as fil-
201 tering high-complexity designs from this pool of constructs could significantly reduce
202 the downstream screening burden. We therefore examine two representative exam-
203 ples of complexity metrics that could potentially be adapted by metabolic engineers
204 for this purpose: one that is primarily derived from network topological analysis and
205 another assessing the breadth and depth of the language used to describe a software
206 program. An extensive review of a range of other metrics in the context of software
207 engineering is given by Zuse [56].

208 Emblematic of software topological analysis is McCabes cyclomatic complexity
209 (CC, [57]) which assesses the complexity of control flow in a software program by
210 representing functions as nodes and control logic connecting them as edges. A large
211 CC value implies more extensive control logic than other programs, while a small
212 CC value indicates that few branch points occur during data processing. However,
213 this approach assumes that both the underlying network topology is human-defined
214 and the flux between nodes is one of information, rather than mass, as is the case for

215 metabolic networks. Given the small number of modified genes in most metabolic
216 engineering designs (Winkler *et al.*, in press), the changes in CC value for most
217 designs compared to the base network will be minute. It may be possible to examine
218 only small, heavily manipulated network modules to calculate a more meaningful
219 topological complexity score.

220 A non-topological alternative to assessing complexity can be found in the Hal-
221 stead metric [58]. This approach examines the number of unique operators (methods
222 for acting on variables) and operands (variables) used in a program, and how many
223 times these are used to define the volume (size), difficulty, and expected amount of
224 programmer effort required for implementation. In the context of metabolic engi-
225 neering, operators are roughly analogous to genetic tools for introducing mutations
226 into strains, while operands can be taken as manipulated genes. Expanding the def-
227 inition of Halsteads metric to include other project facets, such as the motivation
228 behind introducing specific mutations and the number of metabolic network elements
229 manipulated as a consequence of genetic modifications, may be appropriate to better
230 describe the complexity to a given design.

231 Both volume and topology-based metrics have had success in improving several
232 aspects of software engineering. The most important application has been estimating
233 and reducing software maintenance costs [59, 60], as these comprise 40-70% of the
234 total lifecycle cost of a program [59]. Assessment of the ability of software engineers
235 to identify programming errors has also a notable application of these complexity
236 metrics [61, 62]. Security issues can also be anticipated by identifying highly com-
237 plex (i.e. error prone) modules within a software engineering project, allowing costly

238 vulnerabilities to be identified and corrected before they are exploited post-release
239 [63]. Although these applications are unique to software engineering, it is simple to
240 envision analogous roles complexity metrics may play in the context of metabolic
241 engineering: discriminating against high-complexity designs that are difficult to op-
242 timize by accounting for protein, pathway, and genome alterations, evaluating the
243 effect of a library design on the host transcriptome and metabolic network, and
244 estimating the overall cost of metabolic engineering research projects. As the neces-
245 sary standards, data sources, and computational infrastructure become available, we
246 expect that metabolic engineers will begin to explore these applications and more.

247 **Conclusions**

248 In this review, we have examined common sources of complexity at each cellu-
249 lar level and two potential routes for quantifying complexity. Given the increasing
250 interest in developing organism foundries and standardizing organism engineering,
251 efficient filtering of potential designs to identify the least difficult to implement with
252 the highest chance of meeting the desired productivity level is becoming increasingly
253 necessary. Winnowing the set of all possible designs via complexity filtering comple-
254 ments the rapid advances in genome engineering technologies by allowing researchers
255 to eliminate designs that are challenging to construct and optimize without experi-
256 mental screening. As metabolic engineering becomes increasingly standardized, com-
257 plexity analysis tools and guidelines will become an integral part of our engineering
258 toolbox.

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Figures

Figure 1: Distribution of methods used to engineer existing metabolically engineered strains [64]. Legend: HUM, human inference using knowledge or past experience, such as manually examining the metabolic network and selecting mutational targets; LIB, library methods; FBA, flux balance analysis; EVO, adaptive evolution for production or tolerance; RAND, random protein or genome mutagenesis; PREN: targeted protein engineering, TRANS, transcriptomics; LL, liquid-liquid (two-phase) product extraction; COMP, other computational methods; OTH, other approaches.

Figure 2: How protein engineering methods compare in terms of algorithmic complexity and library coverage. Random methods require little (if any) knowledge of the protein of interest but require much larger library sizes to progress through the protein fitness landscape; computational tools are able to reduce the required library size for screening but require substantially more sophisticated algorithms and training datasets to function properly.

Figure 3: Progression in complexity for pathway-level design in metabolic engineering. Initial screens for potential enzymes using high-throughput techniques (metabolomics) that furnish many potentially useful enzymatic reactions; these enzymes can be cloned and expressed using metagenomic libraries. As models become more sophisticated, this random approach can be superseded by metabolic network modeling and database searches of known or predicted pathways.

Figure 4: Complexity trends for genome-level design methods. Random methods, such as mutagenesis and adaptive evolution, are frequently employed in the absence of genotype-phenotype connections. Rational design can be applied if sufficient knowledge and metabolic models are available for the organism in question. Future developments in whole genome refactoring and complete synthesis require significant improvements in our understanding of genome architecture but promise to enable unique engineering approaches.

Meta-Metabolomics

Network Modeling

Pathway Search Algorithms

Algorithmic Complexity and Computation Time