



Synergistic synthetic biology: units in concert

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Synthetic biology aims at translating the methods and strategies from engineering into biology in order to streamline the design and construction of biological devices through standardized parts. Modular synthetic biology devices are designed by means of an adequate elimination of cross-talk that makes circuits orthogonal and specific. To that end, synthetic constructs need to be adequately optimized through *in silico* modeling by choosing the right complement of genetic parts and by experimental tuning through directed evolution and craftsmanship. In this review, we consider an additional and complementary tool available to the synthetic biologist for innovative design and successful construction of desired circuit functionalities: biological synergies. Synergy is a prevalent emergent property in biological systems that arises from the concerted action of multiple factors producing an amplification or cancelation effect compared with individual actions alone. Synergies appear in domains as diverse as those involved in chemical and protein activity, polypharmacology, and metabolic pathway complementarity. In conventional synthetic biology designs, synergistic cross-talk between parts and modules is generally attenuated in order to verify their orthogonality. Synergistic interactions, however, can induce emergent behavior that might prove useful for synthetic biology applications, like in functional circuit design, multi-drug treatment, or in sensing and delivery devices. Synergistic design principles are therefore complementary to those coming from orthogonal design and may provide added value to synthetic biology applications. The appropriate modeling, characterization, and design of synergies between biological parts and units will allow the discovery of yet unforeseeable, novel synthetic biology applications.

Keywords: synthetic biology, synergy, polypharmacology, multi-drug resistance, metabolic engineering, metabolic networks, synthetic lethality, coupling

INTRODUCTION

Synthetic biology is an emerging discipline that proposes the application of engineering principles to design and construct biological systems with innovative and useful functionalities, usually not found in nature. To that end, synthetic biology circuits and devices are basic functional units obtained through the composition of genetic parts that are used in order to build more complex biological systems. Many successful synthetic biology applications have been reported to date, including genetic circuits with specific transfer functions such as oscillators, delayers, inducers, and synthetic metabolic pathways (Wang and Buck, 2012). Due to its engineering roots, the concept of modularity is central to the synthetic biology practice, as modularity is regarded as a key feature that can make biology close to synthetic disciplines such as computer science and engineering (Hartwell et al., 1999; Alon, 2003). Modularity dictates that circuits or devices should be designed in a way that allows their operation to be performed in a modular or plug-and-play fashion, thus allowing the reuse of these modules in different and often unrelated applications. To that end, synthetic biology modules try to satisfy as much as possible the condition of orthogonality, in which modules work as functional units whose intrinsic properties are independent of their environment (Lucks

et al., 2008). Such goal is attained through the adequate attenuation of cross-talk between the elements of the circuits and the host or chassis organism (Del Vecchio et al., 2008). Modules built in that way should meet a standard collection of specifications allowing the application of automated design techniques to synthetic biology devices (Kitney and Freemont, 2012). One of the striking features of synthetic biology applications is their capacity for innovation by implementing new functionalities that were not observed before, at least in their final form, in natural biological systems (Endy, 2005; Lanza et al., 2012). Such innovative behavior is obtained through the rewiring of genetic elements in rather creative ways that often lead to unexpected emergent properties (Benner and Sismour, 2005). Notably, the resulting new behavior can in many cases be regarded as the by-product of a powerful latent property that is present among biological systems, namely the faculty of inducing synergies between constitutive elements toward the emergence of novel or reinforced activities. Even when direct relationships between units are minimized, indirect dependences are still possible to exist in which synergies may arise. Synergy, thus, is a concept inherent to modular systems, which has however not yet been exploited in synthetic biology to the same level of maturity as in other allied fields. By

learning how synergies are unveiled and used, not only in nature but also in many technologically related disciplines such as drug design or metabolic engineering, we might pave the road toward accelerated innovation in synthetic biology applications. Various examples of synergies exist in nature and, in biology, synergy has been recognized as a driving force in evolution (Corning, 2013). Examples of synergies in biology include cooperative interactions between genes or the concerted effect of individuals in bacterial colonies (Wintermute and Silver, 2010). Synergies of scale, functional complementarities, symbiotic relationships, division of labor, etc., are also found everywhere in living systems. Synergies can provide an evolutionary advantage, like in secondary metabolites in plants that act synergistically against pathogens (Challis and Hopwood, 2003), or in the case of the emergence of resistance during multi-drug therapy (Hegreness et al., 2008). Certain genes act in synergy in the expression of a given phenotype. For a set of alleles, synergistic epistatic interaction is defined when the concerted effect of mutants is stronger than the “sum” of the effect of each mutant if considered as independent (Nordwald et al., 2013). Similarly, antagonist epistasis (equivalently alleviating or buffering synergy) is defined when the effect of all mutants together is less severe than the sum of the effect of single mutations (Perez-Pinera et al., 2013). This additive model for null-hypothesis independency is often used when the effect of each mutation can be measured with a log-type scoring such as in measuring drug efficacy for example. Otherwise, multiplicative or min models are used (Mani et al., 2008). In pharmacology, a synergistic/antagonistic drug combination is defined when the efficacy of two drugs is larger/smaller than the sum of the individual effects. The Loewe (additive) and Bliss (multiplicative) models are the corresponding independency models for drug combinations (Cokol et al., 2011).

This review explores how synergies are exploited in several disciplines for a possible adoption of such an approach in synthetic biology applications. The benefits will be multiple, since taking advantage of the synergies found in biological systems will allow us to develop innovative, efficient, and more reliable synthetic biology devices. In the next sections, we will first define first the concept of synergy, giving a formal definition, and then we will look at several techniques used for predicting synergies *in silico*. We will then review important cases of synergies found in drug design, toxicity, and metabolic engineering, as well as the main instances in synthetic biology where synergies were involved as major components in the conception of innovative applications.

THE ROOTS OF SYNERGY IN BIOLOGY

Synergy is a general mechanism that reflects the concerted action of two or several factors on a given outcome of a system. This concerted action produces an amplified or a cancelation (negative amplification) effect compared to the effect produced by each factor individually. Synergy can be defined in multiple ways depending upon the underlying model. In general terms, a property x has synergistic effects on an observed activity $f(x)$ in the system if the dependency of the property with respect to the activity shows superadditivity:

$$f(x_1 + x_2) \geq f(x_1) + f(x_2)$$

Synergy can be in that way measured as the difference between the overall effect and the sum of the individual effects:

$$\text{Syn}(x_1, x_2; f) = f(x_1 + x_2) - (f(x_1) + f(x_2))$$

More interestingly, synergies can also be defined in the context of aggregated effects from a set of elements. Two subsets X_1 and X_2 from a set X display synergistic effects for the activity $f(A)$ when this set function f shows supermodularity (Topkis, 1998):

$$f(X_1 \cup X_2) + f(X_1 \cap X_2) \geq f(X_1) + f(X_2)$$

$$\text{Syn}(X_1, X_2; f) = f(X_1 \cup X_2) + f(X_1 \cap X_2) - (f(X_1) + f(X_2))$$

For instance, the superadditivity and supermodularity models correspond respectively to the Loewe additivity model and Bliss multiplicative models of independent drug effect in drug synergy (Cokol et al., 2011). In an information theory framework, synergy between two factors X_1 and X_2 with respect to the activity f is defined by the difference between the mutual information of each factor simultaneously and independently with respect to f (Anastassiou, 2007):

$$\text{Syn}(X_1, X_2; f) = I(X_1, X_2; f) - (I(X_1; f) + I(X_2; f))$$

In physics, specially in Quantum Electrodynamics (QED) (Feynman, 1988), synergy is described in terms of phase concordance between two events, for instance in the case of light interference, through the concept of amplitudes of probability represented by complex numbers (**Box 1; Figure 1**). Similarly, the phenomena of resonance as seen in spectroscopy or in phase transitions captures the notion of synergy. This coupling phenomenon of resonance is also used in electronics in order to build oscillator circuits. The basic principle consists of establishing a synergy between two filtering elements through a feedback loop that positively amplifies the signal at the frequency of resonance. An oscillator circuit is obtained when the feedback effect cancels the damping effect, so that any small noise perturbation is enough to make the system start to oscillate. Oscillators following that principle are present in many biological systems. Natural oscillators include circadian rhythms, heartbeats, as well as gene expression, metabolic, and neuronal systems (Hess, 2000). Similarly to the electronic circuit oscillator, they are internally controlled through regulatory feedback loops (Bell-Pedersen et al., 2005; Ihekweba et al., 2005), a concept that has been exploited in synthetic gene oscillators by using activator/repressor systems (Atkinson et al., 2003; Stricker et al., 2008; Tigges et al., 2009).

Other examples are the divergence or amplified effect of thermodynamical variables observed in critical phenomena. At phase transitions, a “resonance” can be seen between the macroscopic and microscopic variables at the different length scales present in the system. Nobel laureate physicist by K. Wilson (Kadanoff, 2013) found a way to connect them through a hierarchical framework using a zooming multiscaled parameter (Yeomans, 1992). This concept of scaling has a nice correspondence in biology and is often named as an emergent property. For example, some properties of the lichen emerge when bacteria and microalgae enter in

Box 1 | Synergy in Young's experiments.

In the QED theory, to any given event corresponds an amplitude of probability, a complex number $|r|e^{i\theta}$, the square of which gives the probability of this event to occur. On a complex plane, this complex number is represented by a vector of length $|r|$ and angle θ with respect to the real axis. Two vectors pointing toward the same direction have the same phase. Synergy comes into play when we decompose the event into sub-events. When we add or multiply the amplitudes of probabilities of each sub-event (depending on whether the sub-events happen in alternative or in independent successive ways), the resulting vector may have a greater module if all the vectors of the sub-events point more or less toward the same direction, i.e., when all the vectors have similar phases. The canceling effect arises for example, when an event is decomposed into two alternating (parallel) and equivalent sub-events that are in opposite phases. Such synergistic positive or negative amplification effects could be observed in the famous experiment of light interference by Young (**Figure 1**). The message of this example is that all physical phenomena concerning photons and electrons are explained in QED in terms of amplitude of probability. The complex number view of probability seems to be an ideal tool at least in QED to study synergy.

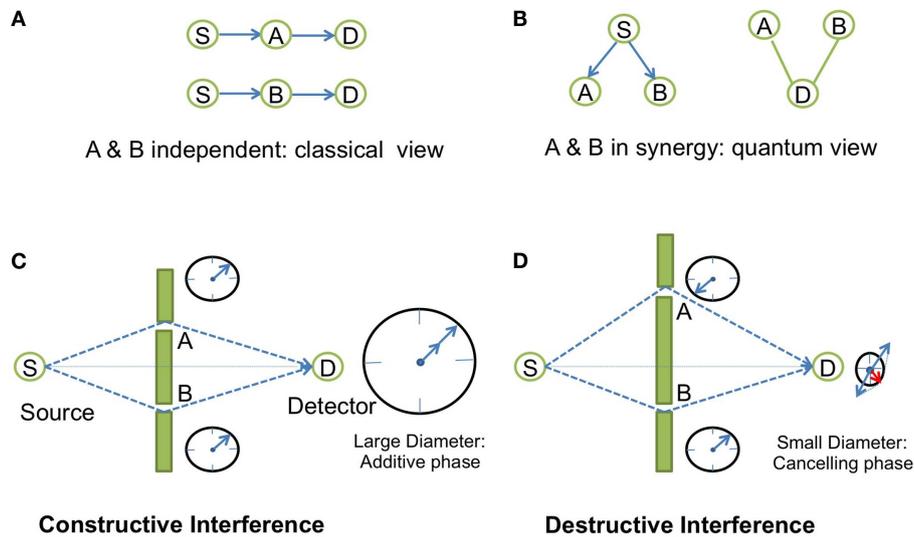


FIGURE 1 | Synergistic effects in Young's interference experiment. In Young's experiment, a source S sends photons to a detector D that is located behind a wall with two tiny slots A and B on each side of the line between S and D . **(A)** When the two slots A and B are independent (by disconnecting the entanglement in measuring the photons emerging from one slot with a detector behind A or B), there is no interference, and the distribution of photons on the z -direction of the screen is just Gaussian. This is the classical view of the experiment. **(B)** In quantum view, using amplitude of probability, the photon emerging from S and going to D has two alternating ways: slot A or slot B . The probability of the total event is the square of the amplitude of probability given by the sum of the two amplitudes for each alternating event $Prob(S \rightarrow D) = |(S \rightarrow A \rightarrow D) + (S \rightarrow B \rightarrow D)|^2$,

where (\cdot) is the amplitude of probability of the event. **(C)** The probability of the total event will correspond to an amplified synergistic effect if the two arrows of the two independent events point toward the same direction, i.e., if the photons are in phase between each other which occurs when the difference between the two trajectories corresponds exactly to an integer number of time period difference between the two photons. This is symbolically represented by a "watch clock" for each photon that turns with a specific speed depending upon the frequency of the light. If the arrows of these two watches point toward the same direction, then the photons are in phase, and we get constructive interferences. **(D)** Destructive interference is observed when the arrows are in the opposite direction (opposite phase).

synergy (symbiosis). Conceptually, this means that at certain conditions of the system the properties of the "forest" do not emerge from studying the properties of the "tree" alone. In biology this scaling property suggests a hierarchical embedding from a single cell to a full organism. Scaling is therefore an additional concept to be integrated in synthetic biology to study, at certain critical conditions, for instance the emergent properties of a population of cells from a single cell.

IN SILICO PREDICTION OF SYNERGIES IN BIOLOGICAL NETWORKS

It is increasingly clear that complex interconnections between components can lead to the emergence of global effects resulting

from a synergy between components or from a concerted ensemble of components called modules. This modular view of biological systems introduces the notion of synergy between modules at a given hierarchical level but also between different hierarchical levels. In analogy with phase transition at critical state, where all length scales are present (Yeomans, 1992), biological systems might reveal synergies between various modular hierarchical levels that explain global biological functions of the entire system (or dysfunctions like in the case of disease states in the organism).

We can think of two types of synergies: the "horizontal" one that corresponds to the concerted actions of modules at a given hierarchical level; and the "vertical" one that can be viewed as a

resonance effect between components or modules at various hierarchical levels. Depiction of synergy depends therefore on the way the biological system is represented. Graph modeling of biological networks captures the relationships between the components, usually at a given length scale. These relationships are usually modeled from the local interactions observed experimentally such as protein–protein interactions or metabolic networks. The goal of the *in silico* model is to unravel the indirect effects or dependencies within this large set of interacting components. *In silico* prediction of synergy is therefore based on two components: (i) hierarchical and/or modular description of the biological network; (ii) implicit or explicit incorporation of the biological response into the model.

Biological response can be included implicitly into the model by constructing for example a disease genes network when looking for a synergy between drug actions (Vitali et al., 2013), or explicitly as for example with a cost function in flux optimization of metabolic networks or as a statistical probability of a functional node in a Bayesian Network approach. Once a biological response is incorporated into the biological network, synergy between components can be deduced directly from the graph topology property of the biological network or by inferring the behavior of conjugate agents on the output response of the system from metabolic fluxes (see Metabolic Synergies below) or Bayesian Network approaches.

The graph-topological approach to explain synergy consists of establishing a relation between the graph property and the biological response in order to define a synergy score from topological graph descriptors. In the case of polypharmacology, the response is modeled through a bi-partite graph and corresponds to the effect of a molecule (drug) on a target or on a disease. This bi-partite graph is made of two kinds of nodes (e.g., drug nodes

and target nodes) with no link between nodes of the same class and can therefore be seen as an interacting graph between the two class of elements, drug and target in this case. In order to deduce the neighborhood description amongst nodes of a same class, like the similarity of behavior of drugs or targets, a co-graph from the bi-partite graph is constructed that connects two nodes of the same class if they share the same partner in the other class. For example, two drugs are connected in the drug-graph if they interact with the same target. Similarly, two targets are connected in the target-graph if they bind a given drug (Yildirim et al., 2007; Li et al., 2011). Li et al. (2011) deduces synergistic drug combinations by analyzing the corresponding disease gene network for that specific disease. Their method, called NIMS (Network target-based Identification of Multicomponent Synergy), calculates drug synergy score using graph-topological descriptors that reflect the type of connection a drug-related gene (node) is making with the rest of the gene network. It includes graph-topological notions like hubs and betweenness centrality (Li et al., 2011; Vitali et al., 2013). This approach has been tested on a set of five agent pairs with known synergy in every 62 pairs for a given agent in the therapeutic area of angiogenesis and rheumatoid arthritis.

Another topological approach presented by Vitali et al. (2013) proposes an efficient identification of multicomponent synergies that rely on the particular topological properties of the nodes (Disease-Protein) in the disease network that are constructed from protein–protein interactions and expression data for the diseases under study. The selection of potential protein candidates for pharmaceutical synergy is based on topological properties of the network (Table 1; Figure 2). Then, a set function called Topological Score of Drug Synergy (TSDS) assigns a score to each combination of secondary proteins. Such a score is computed for

Table 1 | Synergy score estimation from network topological properties.

Type of score	Definition	Example of application
Bridging centrality	$BR(p) = B(p) \times BC(p)$ <p>$B(p)$ is the betweenness centrality given by the fraction of shortest paths between node pairs that pass through the node p</p>	Network-based characterization of drug-related genes (Kotlyar et al., 2012)
Bridging coefficient	$BC(p) = \frac{d(p)^{-1}}{\sum_{v \in N(p)} d(v)^{-1}}$ <p>Discriminates if a node p connects hub nodes, based on the degree $d(p)$ of neighbors $N(p)$</p>	Network identification of robust bridged nodes (Hwang et al., 2008)
Node reachability index	$DP(p) = \frac{\sum_{d=1}^{N_d} \sum_{i=1}^{N_{sh}(p,d)} \prod_{i,j} w_{ij}}{N_{sh}(p)}$ <p>Sum of the weights w_{ij} of each shortest pathway between the source protein p and the diseases d divided by the number of pathways N_{sh}</p>	Network-based prioritization of drug targets (Chua et al., 2011)
Topological score of drug synergy	$TSDS(p_1, p_2, p_3) = \prod_{p \in (p_1, p_2, p_3)} DP(p)$ <p>Product of the reachability index $DP(p)$ for each triplet of source proteins (p_1, p_2, p_3)</p>	Network-based target ranking for polypharmacological therapies (Vitali et al., 2013)

METABOLIC SYNERGIES: PATHWAY COMPLEMENTARITIES

Synergies are often found in organisms at the level of metabolism. Observed metabolic phenotypes in the organism's metabolic network can be seen as emergent properties resulting from the tradeoff balance between competing objectives such as growth and energy consumption under Pareto's optimality condition (Schuetz et al., 2012). Perturbations in the network will induce adjustments in order to achieve optimality under the new conditions. Synergies appear when the simultaneous perturbation of two nodes in a metabolic network induces a readjustment of flux distributions that corresponds to an augmented effect at a distant site in the network. Identifying such types of effects through FBA can be used either to design metabolic interventions increasing the production of a desired target metabolite in metabolic engineering or, in the opposite direction, to disrupt the metabolism of a pathogen or a cancer cell by multi-target and synthetic lethal drugs (Box 2). Synergies can also be observed at the systems level in the concerted operation of metabolic networks, like in the case of symbiotic and parasitic relationships (Christian et al., 2007) or in ecosystems (Klitgord and Segrè, 2011), where the metabolism of different species act in cooperation.

As discussed in the previous section, synergies are studied through metabolic networks in order to identify combinations of drug targets that have increased efficacy. For instance, compounds that inhibit enzymes in the pathway can target multiple enzymes in the pathway, since often they have conserved binding motifs interacting with similar metabolites acting as intermediates. This was the case for the shikimate pathway of *Helicobacter pylori*, where through a screening procedure compounds were found that could target both the shikimate dehydrogenase and shikimate kinase (Hsu et al., 2013). Studies of synthetic lethality can be performed *in silico* through FBA. In a reconstructed model of *Mycobacterium tuberculosis*, all pairs of double-deletion mutants (synthetic lethal pairs) were tested through FBA. Drugs associated with such synthetic lethal antimicrobial targets can represent drug synergy (Chavali et al., 2012). Similarly, the observation that many cancer cells adapt their metabolism has led to the identification of synthetic lethal drugs through FBA of metabolic networks; these synergies were thereafter validated using available drug efficacy and gene expression measurements (Folger et al., 2011; McCarthy, 2011).

Box 2 | Finding synergies in metabolic engineering.

In flux balance analysis (FBA), metabolic reactions are represented as a stoichiometric matrix \mathbf{S} , where each row represents a compound and each column represents one reaction. The flux through the reactions in the network is represented by \mathbf{v} and it is constrained between v_i^{\min} and v_i^{\max} . FBA estimates the optimal flux solution that maximizes an objective function v_{bio} such as biomass, energy consumption, etc., expressed as a combination of fluxes, among the allowable space of flux distributions (Orth et al., 2010):

$$\begin{aligned} &\text{maximize } v_{\text{bio}} \\ &\text{subject to} \\ &\mathbf{Sv} = \mathbf{0} \\ &v_i^{\min} \leq v_i \leq v_i^{\max} \end{aligned}$$

The solution to the previous constraint-based optimization can be found through the application of linear programming algorithms. Interestingly, in order to find synergies between nodes in the previous network that maximize (or minimize) the flux, v_x , associated with the desired activity, the optimization problem can be augmented into a mixed integer linear programming (MILP) problem where pairs, triplets, and so on of gene deletions are tested in order to find the best synergy that simultaneously maximize v_x and v_{bio} , stated as follows:

$$\begin{aligned} &\text{maximize } v_x \\ &\text{subject to} \\ &\left[\begin{array}{l} \text{maximize } v_{\text{bio}} \\ \text{subject to} \\ \mathbf{Sv} = \mathbf{0} \\ v_i^{\min} \leq v_i \leq v_i^{\max} \end{array} \right] \\ &\sum_i (1 - y_i) = K, \quad y_i = \{0, 1\} \end{aligned}$$

where K is the number of allowable deletions. Depending upon the type of synergy (positive or negative), two types of applications are at least possible:

1. In synthetic lethality studies, v_x is a negative flux that corresponds to the synergistic drug target, for instance in order to determine essential genes that impair growth in cancer cells (Folger et al., 2011).
2. In metabolic engineering, the goal is to introduce metabolic interventions such as gene knockouts that modify the fluxes so that the flux v_x through the desired final product is maximized (Shen and Liao, 2013).

Synergies in metabolic networks are also a valuable tool for metabolic engineering. One of the implicit objectives of metabolic engineering is to employ synergies in order to achieve optimal production of a desired metabolite. Such synergies between metabolic pathways arise when the requirements of the pathways, such as energy, redox, or cofactor exchanges, are complementary (Shen and Liao, 2013). To that end, network analysis can be performed in order to determine simultaneous gene knockouts, knockdowns, and overexpression (knock-ins) in the host organism leading to overproduction of the target. For instance, a synergistic effect was observed on the production of S-adenosyl-L-methionine in *Pichia pastoris* by simultaneously using knocking in and knocking out techniques (He et al., 2006). In another example, a synergistic increase in plant oil levels was attained by simultaneously engineering genes involved in the biosynthesis of fatty acids (Vanhercke et al., 2013).

Such studies are based on the estimation of changes in the equilibrium of fluxes through FBA when multiple genes encoding enzymes are deleted in the network (Ranganathan et al., 2010). In addition, synergies in metabolic engineering can also be obtained through synergistic fine control of gene expression of the enzymes in the pathway in order to allow the reduction of bottlenecks and of growth inhibition from accumulation of intermediates (Flowers et al., 2013). Developing regulatory parts such as promoters, ribosome binding sites (RBS), and riboswitches have been shown to be useful for achieving such synergies (Arpino et al., 2013). Furthermore, another type of synergy that is naturally found in metabolic pathways are the ones induced by enzyme co-localization when reactions are brought into close proximity through a multifunction enzyme that couples sequential conversion steps in the pathway, allowing by this means substrate channeling. Benefits are multiple; namely, enzyme co-localization reduces intermediate's diffusion distance, can keep local concentrations high, and can reduce cross-talk between competing pathways. Many examples exist where synergistic improvements in comparison with individual enzyme activities were obtained through co-localization (Conrado et al., 2008). Interestingly, this strategy has been mimicked by engineers in the design of synthetic protein scaffolds that physically

link enzymes in a way that allowed the effective concentration of each component of the desired pathway to be increased (Dueber et al., 2009).

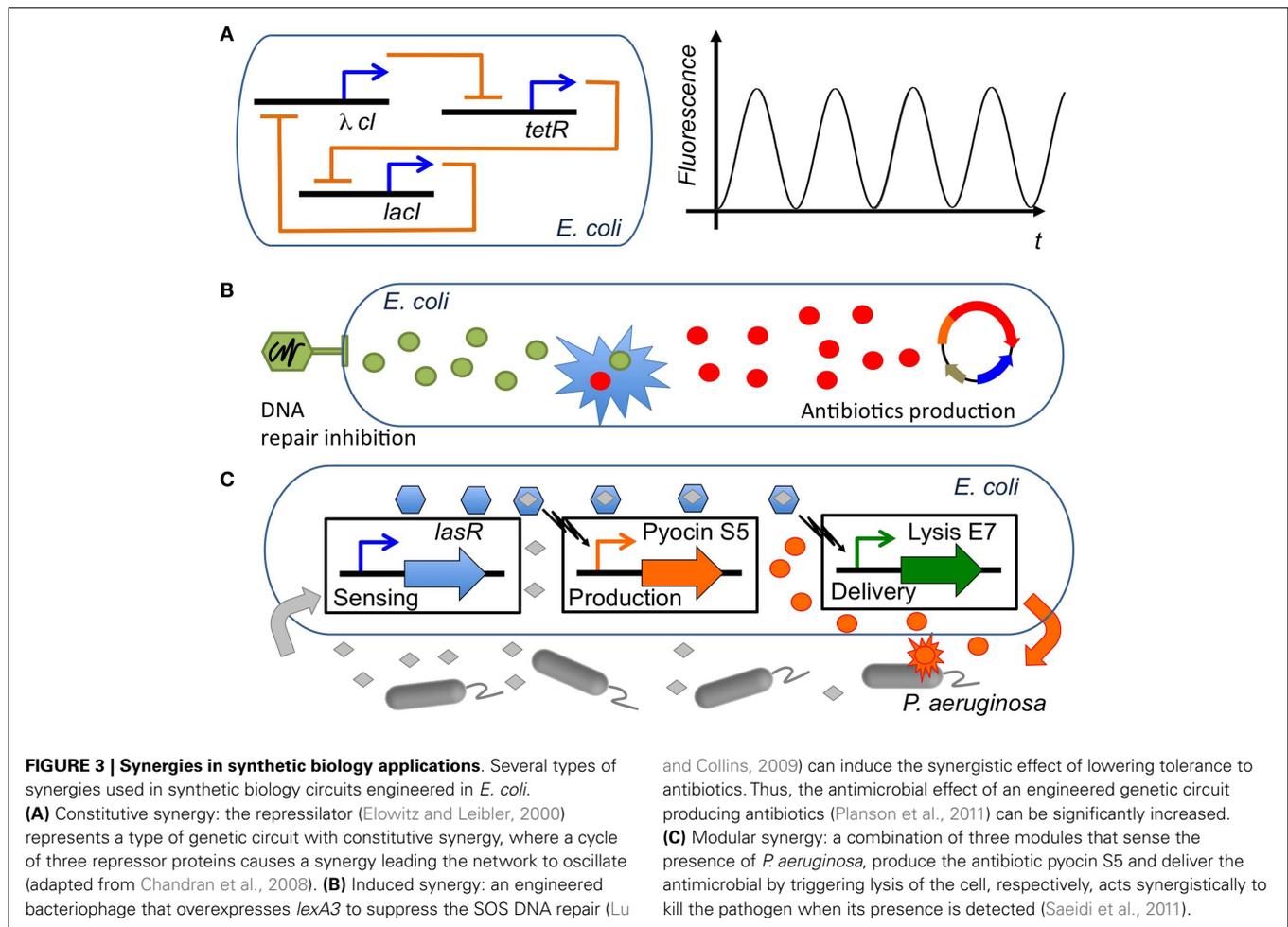
SYNERGISTIC DESIGN FOR SYNTHETIC BIOLOGY INNOVATION

Synergy is a concept that permeates much of synthetic biology. Although in a rather non-systematic way, synthetic biology has often taken advantage of existing synergies, either natural or those found in experimental screening, in order to create new behavior from the combination of biological elements (Agapakis and Silver, 2009; Khalil and Collins, 2010). Synergies in synthetic biology applications can be grouped into three main types (**Table 2**; **Figure 3**): (i) constitutive synergies, which are found when the emergent behavior of the device comes from synergies among its internal elements; (ii) induced synergies, which occur if the output activity of two or more synthetic biology devices induce a synergistic effect in a host or external organism; (iii) modular synergies, which appear when the independent behavior of multiple modules gives birth to a synergistic outcome once they are interconnected.

Constitutive synergy is established between parts of a synthetic biology module in order to provide its desired function. They are obtained by the skillful application of genetic circuit design principles. Such cooperative interactions among genetic parts enabled many of the early synthetic biology devices. Among others, toggle switches (Gardner et al., 2000) and oscillator gene circuits such as the repressilator (Elowitz and Leibler, 2000) were early examples of gene cooperativity giving birth to a well-defined emergent behavior. The resulting cooperative behavior between genes is most often linked to feedback relationships (To and Maheshri, 2010; Jayanthi et al., 2013), i.e., genes that are combined through repression/activation interconnections in a way that allows generation of the desired synthetic behavior (Rollie et al., 2012). In order to establish a synergistic effect between the genes, properties for each part need to be carefully selected. For instance, it has been shown that to obtain oscillations, it is necessary to impose a time-scale design condition with a repressor that is sufficiently slow in comparison with the activator (Jayanthi and Del Vecchio, 2012). Once

Table 2 | Examples of synthetic biology synergistic applications.

Type of synergy	Elements showing synergistic effects	Main analysis and design methodologies	Synthetic biology applications
Constitutive	Circuit's genetic parts	Genetic circuit design	Toggle switch (Gardner et al., 2000) Repressilator (Elowitz and Leibler, 2000)
Induced	Organism's biological elements (drugs, mutations, pathways, etc.)	Systems biology	Combinatorial antibiotic treatment (Lu and Collins, 2009; Weber and Fussenegger, 2009; Kohanski et al., 2010)
		Network analysis Metabolic network analysis	Synergic treatment of metabolic disorders (Ye et al., 2013) Bistability in bacterial populations (Tan et al., 2009)
Modular	Synthetic modules	Biosensor design	Auxin biosensor from a mammalian and a plant circuit (Wend et al., 2013)
		Interface design	Sensor and delivery for killing pathogens (Saeidi et al., 2011)
		Systems and control theory	Analog (Daniel et al., 2013) and multicellular computation (Gofñi-Moreno et al., 2013)



a constitutive synergy has been appropriately designed and tuned in the circuit, the resulting functional device can potentially be employed in multiple synthetic biology applications.

A second level of synergy used in synthetic biology is induced synergy, i.e., synergy that is created between the outputs of synthetic biology devices. As discussed in previous sections, many useful synergistic events in biological systems have been identified through systems biology and network analysis. Synthetic biology modules are here used in cooperation in order to trigger external synergistic events (either in the host organism or in an external pathogen) that might be useful for biological applications. For instance, to this category belong applications that use drug synergies through the delivery of multiple drugs by synthetic biology devices. Essential genes appear here as a basic tool used in order to identify synergistic targets, for example with antimicrobial activity (Juhas et al., 2012). One example is the use of engineered bacteriophages to attack gene networks that are not directly targeted by antibiotics (Lu and Collins, 2009; Lu et al., 2009). By modifying the oxidative stress response or the inhibition of DNA damage repair systems in *Escherichia coli*, the antimicrobial effect of several antibiotics such as quinolone, gentamicin, and ampicillin was enhanced. Furthermore, control of biofilm formation and inhibition of mechanisms involved in antibiotic resistance were also

enhanced by the engineered bacteriophages (Weber and Fussenegger, 2009; Kohanski et al., 2010). In yet another example, a first molecule (guanabenz) was used in order to activate a synthetic signal cascade that stimulated the secretion of a fusion protein that regulates the metabolism based on the synergistic action of a peptide (GLP-1) and the hormone leptin (Ye et al., 2013). The authors showed that it was by this means possible to regulate the metabolic syndrome states linked to several diseases (obesity, hypertension, hyperglycemia, etc.). Besides these examples of induced synergy between the outputs of synthetic biology circuits, other induced synergies might appear between the inserted circuit and the host organism because of variations in global host resources (Cardinale et al., 2013) or cell growth (Scott et al., 2010), leading to different circuit performances. Interestingly, such synergistic effects have been used to generate bistability in bacterial populations through growth-rate dependent gene expression (Tan et al., 2009; Klumpp, 2011), opening the possibility to another type of synthetic biology applications based on the induced synergy between circuits and the host organism.

In the third group of synthetic biology synergies belong those that emerge at the modular level, i.e., emergent behavior elicited by the concerted operation of multiple synthetic biology devices. If the two previously described groups of synergy were established

either internally (constitutive) or externally (induced) with respect to the inserted synthetic biology modules, modular synergy is a synergy that is specifically established between the modules. Here, each synthetic biology module operates in a rather independent or orthogonal way from the others except at the input/output interface where the actual synergy is formed. The resulting circuit interferences or cross-talk can be characterized through properties such as retroactivity, a property analog to output impedance in electronic circuits (Del Vecchio et al., 2008; Jiang et al., 2011), and fan-out (Kim and Sauro, 2010). Theoretical studies have shown that these properties can be finely tuned through an appropriate insulation and time-scale separation (Jayanthi and Del Vecchio, 2011). For instance, insulation mechanisms for module interfaces have been proposed based on phosphorylation-dephosphorylation cycles (Del Vecchio et al., 2008), the appropriate selection of DNA target sites for transcription factors (Jayanthi and Del Vecchio, 2012) or through the signaling metabolite acetyl phosphate in order to generate oscillations (Fung et al., 2005). In general, introducing delays at the circuit's interface through transcription and translation control (riboregulators, RBS, etc.) (Purnick and Weiss, 2009), regulatory regions, degradation, inducible promoter regulation (Jayanthi and Del Vecchio, 2012) or enzymatic processes (Cookson et al., 2011) constitute flexible methods to tune synthetic biology circuits. These genetic elements work very often in a synergistic way (Perez-Pinera et al., 2013). Therefore, models that precisely estimate synergistic activities are needed in the synthetic biology design toolbox. Some examples are operon calculators (Salis et al., 2009) as well as others ways of controlling gene expression from genetic elements (Meng et al., 2013; Mutalik et al., 2013). Synthetic biology applications that have used modular synergies include for instance an *E. coli* strain that was engineered to sense and kill *Pseudomonas aeruginosa*. A first module consisted of a sensing device that detected the presence of *P. aeruginosa* through its quorum sensing signal. A second module was responsible for the production of pyocin, an antibiotic and of E7 lysis protein causing the chassis to lyse and resulting in the release of pyocin (Saeidi et al., 2011). In this application, the concerted operation of both modules resulted in the killing activity.

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In another example, a synergistic mammalian and plant synthetic biology approach was used to develop a ratiometric luminescent biosensor with wide applications including study of auxin metabolism, transport, and signaling (Wend et al., 2013). More advanced applications of modular synergy are circuits performing complex computations, such as synthetic analog gene circuits (Daniel et al., 2013) and multicellular computation (Goñi-Moreno et al., 2013).

PERSPECTIVES

Synergies have been driving innovation both in nature and in synthetic designs. They have served to implement innovative solutions in fields as diverse as drug design, where drug synergies provided new ways to overcome urgent problems such as resistance, and in metabolic networks, where synergies have found their way in pathway complementarity toward amplified targeted effects. By pushing forward the concept into synthetic biology, we would argue that new promising applications may emerge in the near future.

To date, synergy has already been successfully used in synthetic biology to induce non-natural behavior in synthetic constructs, to empower the outcome of biological circuits, or to enable the concerted operation of modules into an emergent function. The next frontier will be in the development of a systematic characterization of synergistic effects in synthetic biology devices in a similar manner as synergies are characterized through modeling and screening in other disciplines such as drug design and metabolism. Beyond the concept of orthogonality in modular design, synergistic cross-talk between modules arises here as a valuable feature that should be appropriately characterized in the specifications found in biological circuit catalogs. The adoption of such proposed practice of synergistic synthetic biology, thus, will require a design paradigm shift toward circuit topologies that will deliberately display the ability of emergent behavior once operating in concert.

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