A Control Theoretic Framework for the Analysis and Design of Biological Networks

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A Control Theory Approach to Engineering Biomolecular Networks

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Abstract: Control theory has been instrumental for the development of a number of engineering systems, including aerospace and transportation systems, robotics and intelligent machines, manufacturing chains, electrical, power, and information networks. In the past decade, the ability of engineering biomolecular networks has become a reality in the rising field of synthetic biology. Biomolecular networks are composed of repression and activation interactions among genes, proteins, and small signaling molecules, and have the potential of implementing arbitrarily complex functions. While modular analysis and design is a promising approach to engineer complex networks, it is still subject of debate whether a modular approach is viable in biomolecular systems. The dynamics of these networks are highly nonlinear and therefore addressing this question requires the use of tools from nonlinear control theory. Here, we review some of the techniques that we have been developing in order to analyze and design biomolecular networks modularly.

1. INTRODUCTION

The past decade has seen tremendous advances in the fields of systems and synthetic biology to the point that *de novo* creation of simple biomolecular networks, or "circuits", that control the behavior of living organisms is now possible (Baker et al. [2006]). A near future is envisioned in which re-engineered cells will perform a number of useful functions from turning waste into energy, to killing cancer cells in ill patients, to detecting pathogens in the environment. To meet this vision, one key challenge must be tackled, namely designing biomolecular networks that can realize substantially more complex functionalities than those currently available (Atkinson et al. [2003], Elowitz and Leibler [2000], Gardner et al. [2000]). This ability is still missing.

A promising approach to designing or analyzing complex networks is to connect simple modules whose behavior can be isolated to some extent from that of the surrounding modules. The key assumption in this approach is that the behavior of a module does not change upon interconnection. This is often taken for granted in fields such as electrical engineering, in which amplifiers enforce modular behavior by suppressing impedance effects. Whether a modular approach is viable in biomolecular networks is still subject of intense debate (Hartwell et al. [1999], Purnick and Weiss [2009]). Here, we address this fundamental question by illustrating how impedance-like effects are found in biomolecular systems, just like in many engineering systems. These effects, which we call retroactivity (Del Vecchio et al. [2008]), can be severe and alter the behavior of a module upon interconnection, undermining modular behavior. By employing tools from singular perturbation theory, we provide an operative quantification of retroactivity as a function of biomolecular parameters and network topology (Gyorgy and Del Vecchio [2012]). Specifically, we determine interconnection rules that account for retroactivity by calculating equivalent retroactivities, just like Thevenin's theorem does for electrical circuits (Agarwal and Lang [2005]). This way, we recover the predictive ability of a modular approach to design.

When designing systems, it is often desirable that the behavior of a module in isolation is not altered when it is connected to other modules. In order to reach this objective, we have proposed to interconnect modules through insulation devices, which buffer modules from retroactivity effects (Jayanthi and Del Vecchio [2011]). By merging disturbance rejection and singular perturbation techniques, we provide an approach that exploits the distinctive structure of biomolecular networks to design biomolecular insulation devices. We illustrate the application of this approach through an implementation based on protein covalent modification cycles (Jiang et al. [2011]). Specifically, we illustrate that covalent modification cycles, ubiquitous in natural signal transduction, can be re-engineered to function as insulation devices for synthetic biology applications.

In Section 2, we introduce the modular composition problem through a motivating example. In Section 3, we introduce the retroactivity concept and illustrate how to apply singular perturbation theory to obtain an operative quantification of retroactivity effects. Section 4 formulates the problem of designing insulation devices as a disturbance attenuation problem and tackles it by using singular perturbation theory.

2. MOTIVATING EXAMPLE

As a motivating example, consider the biomolecular activator-repressor clock of Atkinson et al. [2003] showed in Figure 1(a). This oscillator is composed of an activator A activating itself and a repressor R, which, in turn, re-

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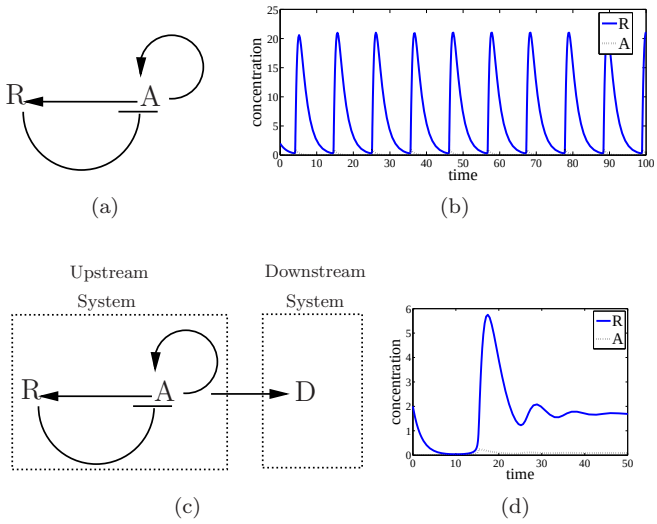

Fig. 1. (a)-(b) show the activator-repressor clock topology and the time behavior of the activator and repressor concentrations. (c)-(d) show that when a load is connected to the clock, sustained oscillations disappear. In all simulations, we have chosen $K_a = K_r = 1$, $\alpha_A = \alpha_R = 100$, $\alpha_{0,A} = 0.01$, $\alpha_{0,R} = 0.004$, $\delta_A = 1$, $\delta_R = 0.5$, $n = 2$, and $m = 4$ (model (1). In (d), we also have $k_{\text{on}} = k_{\text{off}} = 50$ and $p_T = 5$ (model where A dynamics modify to (2)).

presses the activator A. Both activation and repression occur through transcription regulation (Alon [2007]). Specifically, denoting in italics the concentration of species, the model of this clock can be written as

$$\begin{aligned} \dot{A} &= \frac{\alpha_A (A/K_a)^n + \alpha_{0,A}}{1 + (A/K_a)^n + (R/K_r)^m} - \delta_A A, \\ \dot{R} &= \frac{\alpha_R (A/K_a)^n + \alpha_{0,R}}{1 + (A/K_a)^n} - \delta_R R, \end{aligned} \tag{1}$$

in which δ_A and δ_R represent protein decay (due to dilution and degradation). The functions $\frac{\alpha_A (A/K_a)^n + \alpha_{0,A}}{1+(A/K_a)^n+(R/K_r)^m}$ and $\frac{\alpha_R (A/K_a)^n + \alpha_{0,R}}{1+(A/K_a)^n}$ are Hill functions (Alon [2007]), in which the first one increases with A and decreases with R while the second one increases with A. It was shown in Del Vecchio [2007] that the key mechanism by which this system displays sustained oscillations is a Hopf bifurcation with bifurcation parameter the relative time scale of the activator dynamics with respect to the repressor dynamics. Specifically, as the activator dynamics become faster than the repressor dynamics, the system goes through a supercritical Hopf bifurcation and a periodic orbit appears (Figure 1(b)).

When one wants to consider the clock as a signal generator to be used to, for example, time or synchronize downstream systems, ideally one would take one of the proteins of the clock, say protein A, as an input for a downstream system (Figure 1(c)), in which A will activate the expression of another protein D, for example. In this case, one needs to add to the clock dynamics the description of the physical means by which information is transmitted from the upstream system to the downstream one. In any biomolecular system, information is transmitted through (reversible) binding reactions. In this case, A will reversibly bind with the promoter p controlling the

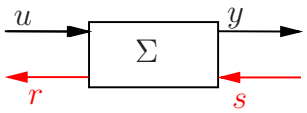

Fig. 2. System concept with retroactivity (Del Vecchio and Sontag [2009]).

expression of protein D to form a complex C. Letting p_T denote the total concentration of this promoter and k_{on} and k_{off} the association and dissociation rate constants, we have that the A dynamics modify to

$$\begin{aligned} \dot{A} &= \frac{\alpha_A (A/K_a)^n + \alpha_{0,A}}{1 + (A/K_a)^n + (R/K_r)^m} - \delta_A A \\ &\quad - k_{\text{on}} A (p_T - C) + k_{\text{off}} C, \\ \dot{C} &= k_{\text{on}} A (p_T - C) - k_{\text{off}} C, \end{aligned} \tag{2}$$

while the differential equation for R remains the same. Since A is an activator of D, we will also have that $\dot{D} = kC - \delta_D D$, in which k and δ_D are the production rate constant and the decay rate constant, respectively. As a result of this interconnection, the clock stops functioning (Figure 1(d)). This effect has been called *retroactivity* to extend the notion of loading or impedance to non-electrical systems, and in particular to biomolecular systems (Del Vecchio et al. [2008]). It is due to the fact that the communicating species, A in this case, when busy in the reactions of the downstream system cannot participate in the reactions of the upstream system and hence the clock behavior is affected. More details on how biomolecular clocks are influenced by retroactivity can be found in Jayanthi and Del Vecchio [2012]. In the next sections, we illustrate a systems theory framework to explicitly model retroactivity to make the problem of retroactivity amenable of theoretical study.

3. RETROACTIVITY

In order to model retroactivity, we propose to model systems as shown in Figure 2. Specifically, we explicitly add retroactivity as signals traveling from downstream to upstream. Signal s is the retroactivity to the output and models the fact that whenever the output y of Σ becomes an input to a downstream system, this system affects the upstream system dynamics because of the physics of the interconnection mechanism. Similarly, r is called the retroactivity to the input and models the fact that whenever Σ receives signal u, it changes the dynamics of the sending system. Related system concepts include that of Paynter [1961] and that of Polderman and Willems [2007]. Differently from Paynter [1961], our framework does not require that signals r and u (s and y) are generalized effort and flow variables and hence that their product is the power flowing through the port. Differently from Polderman and Willems [2007], we keep a directionality to these signals as we seek to identify upstream-to-downstream as the direction in which we think information is being transmitted. From a practical point of view, this is useful because a module is usually characterized by forcing input signals and measuring the consequent output signal. So, there is an intrinsic directionality already associated with the information transfer within a module.

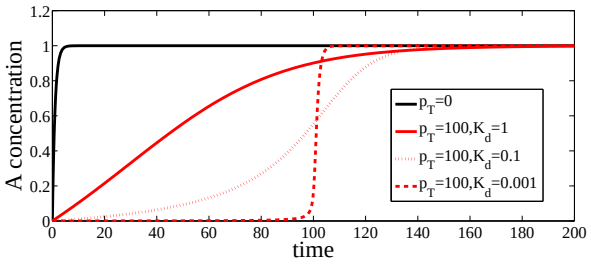

Fig. 3. Effect of retroactivity on the dynamics of A when subject to constant production rate $K(t) = 1$.

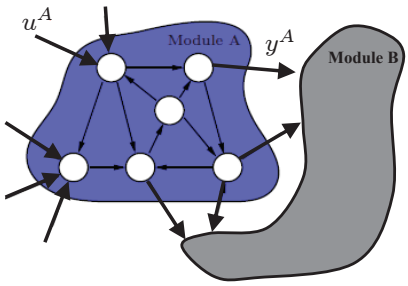

Fig. 4. Network A connects to network B.

Because of retroactivity, the connected behavior of a module differs from the behavior of the same module in isolation ($s = 0$). We seek to predict how the behavior of a module will change upon interconnection as a function of the biochemical parameters characterizing the interconnection. As an example, consider the dynamics of A in the clock when the clock is connected to downstream systems:

$$\begin{aligned}\dot{A} &= K(t) - \delta_A A - k_{\text{on}} A(p_T - C) + k_{\text{off}} C,\\ \dot{C} &= k_{\text{on}} A(p_T - C) - k_{\text{off}} C,\end{aligned} \tag{3}$$

in which, we have denoted $K(t) = \frac{\alpha_A (A/K_a)^n + \alpha_{0,A}}{1+(A/K_a)^n+(R/K_r)^m}$ as its specific form is not relevant for the current discussion. In this system, we have that $s = k_{\text{on}} A(p_T - C) - k_{\text{off}} C$ is the retroactivity to the output and it is conceptually similar to a flow. How is this flow affecting the A dynamics when compared to the isolated one $\dot{A} = K(t) - \delta_A A$? To answer this question, we can exploit the natural time-scale separation between protein production and decay (δ_A), in the order of minutes to hours, and binding/unbinding rates (k_{off}), in the order of seconds/subseconds (Alon [2007]). By defining the small parameter $\epsilon = \delta_A / k_{\text{off}}$ and the slow variable as $z = A + C$, system (3) can be taken to standard singular perturbation form (Khalil [2002]). One can show that the slow manifold is locally exponentially stable (Del Vecchio et al. [2008]), so that the reduced system takes the form

$$\dot{A} = (K(t) - \delta_A A)\left(\frac{1}{1+\mathcal{R}(A)}\right),\ \mathcal{R}(A) = \frac{p_T/K_d}{(A/K_d+1)^2}, \tag{4}$$

in which $K_d = k_{\text{off}}/k_{\text{on}}$ is the dissociation constant of the binding between A and the promoter p. The expression of $\mathcal{R}(A)$ provides an operative quantification of retroactivity as function of the relevant parameters. Specifically, retroactivity increases when p_T increases (the load increases) and/or K_d decreases (the affinity of the binding increases), which is physically intuitive. Furthermore, since $\mathcal{R}(A) > 0$, we have that equation (4) implies that the dynamics of A slow down in the connected system configuration. As K_d becomes smaller, this "slow down" becomes close to a finite-time delay (Figure 3). Since retroactivity slows down the dynamics of the output species, it is natural that the clock in Section 2 stops oscillating if the load is sufficiently high. In fact, the addition of the load causes the activator dynamics to slow down compared to the repressor dynamics and hence the system moves to the "left" of the supercritical Hopf bifurcation so that the equilibrium point becomes stable. At this point, one may ask the question of how the clock behavior would be affected if as communicating species one chooses the repressor. Since retroactivity slows down the dynamics of the repressor, one should expect that a non-oscillating clock can be turned into an oscillating clock for sufficiently high load as the system moves through the Hopf bifurcation. This was formally shown in Jayanthi and Del Vecchio [2012]. The effects of retroactivity on the behavior of biomolecular systems have been experimentally verified both *in vivo* (Kim et al. [2010], Jayanthi et al. [2013]) and in signaling systems *in vitro* (Jiang et al. [2011]).

The operative quantification of retroactivity can be extended to the interconnection of any two general networks (Figure (4)). In particular, given the isolated modules dynamics $\dot{x}^A = f_0^A(x^A, u^A)$ and $\dot{x}^B = f_0^B(x^B, u^B)$, in which x^A, x^B, u^A, u^B are vectors with $\dim(y^A) = \dim(u^B)$, one can demonstrate that the dynamics of connected module A will have the form

$$\begin{aligned}\dot{x}^A = (I + (I + R^A)^{-1} S^B)^{-1} (f_0^A(x^A, u^A) + \\ (I + R^A)^{-1} M^B f_0^B(x^B, y^A)),\end{aligned} \tag{5}$$

in which R^A, S^B, and M^B are state-dependent matrices, which depend only on measurable parameters, such as promoter amounts and dissociation constants, and on the interconnection graph (see Gyorgy and Del Vecchio [2012] for more details). Matrix R^A is called *internal retroactivity* and quantifies the effect of intramodular connections on the dynamics of module A in isolation. Matrix M^B is called the *mixing retroactivity* and quantifies the "coupling" between the isolated dynamics of module A and the isolated dynamics of module B. Specifically, when $M^B = 0$, the isolated dynamics f_0^B of module B do not appear in the dynamics of module A, so that the two dynamics are not "mixed". From a physical point of view, this mixing occurs when nodes in module B have parents from both module B itself and module A, so that transcription factors from A and B interfere with each other while binding to promoter sites in module B. When $M^B = 0$, the dynamics of module A are simply given by

$$\dot{x}^A = (I + (I + R^A)^{-1} S^B)^{-1} f_0^A(x^A, u^A), \tag{6}$$

so that the dynamics of module A are a "matrix-scaled" version of the dynamics of A in isolation. This is why matrix S^B is called the *scaling retroactivity* and quantifies the loading effect that module B has on module A due to transcription factors in A binding to promoter sites in module B. When also $S^B = 0$, the dynamics of module A are the same as in isolation. From a conceptual point of view, the internal retroactivity R^A plays a similar role to the equivalent (according to Thevenin's theorem) output admittance of module A in the electrical case, while S^B plays a similar role to the input admittance of module B. This analogy is purely conceptual since the structure of the two systems (gene network and electric network) is fundamentally different especially since one

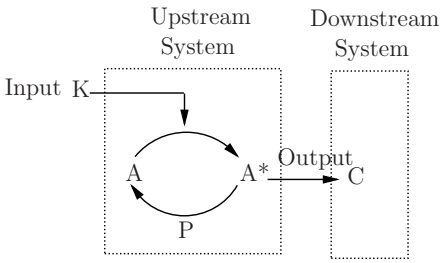

Fig. 5. A phosphorylation cycle is a protein modification mechanism in which an inactive protein A is converted by a kinase K to an active form A*, which is converted back to A by a phosphatase P.

(the electrical network) is linear while the other (gene network) is nonlinear.

4. INSULATION

From a design point of view, it is often desirable that the behavior of the upstream system does not change when it is connected to a downstream one. However, we have seen that retroactivity causes a potentially dramatic change in the dynamics of the upstream system. If one cannot design the downstream system to have low scaling and mixing retroactivity, a viable option is to design a device that once placed between module A and B insulates them from retroactivity effects. We call this device an *insulation device* and we formally specify its properties by requesting that it is a system Σ as in Figure 2, in which (a) $r \approx 0$ and (b) the effect of s on y is completely attenuated. The second requirement is particularly interesting since it can be formulated as a disturbance attenuation problem, as we next explain.

One well established technique for disturbance attenuation is high-gain feedback. We illustrate how this idea applies to our problem by considering again the dynamics of A in the isolated $\dot{A} = K(t) - \delta_A A$ and connected $\dot{A} = (K(t) - \delta_A A)(1/(1+\mathcal{R}(A))$ upstream system configuration. In this case, the idea of high-gain feedback is to apply a negative feedback gain G and, in order not to attenuate the signal $A(t)$, to apply a similarly large gain $G' = \alpha G$ for some $\alpha > 0$ to the input $K(t)$. In this case, the dynamics of the isolated and connected system become

$$\begin{aligned} \dot{A} &= G'K(t) - \delta_A A - GA \text{ (isolated)} \\ \dot{\bar{A}} &= (G'K(t) - \delta_A \bar{A} - G\bar{A}) \left(\frac{1}{1+\mathcal{R}(\bar{A})}\right) \text{ (connected)} \end{aligned} \quad (7)$$

so that when G increases, we have that $|A(t) - \bar{A}(t)| \to O(1/G)$ (Del Vecchio and Sontag [2009]), which implies that the effect of retroactivity on $\bar{A}(t)$ is attenuated as G increases. What type of biomolecular system can implement an input amplification and a similarly large negative feedback? Consider a phosphorylation cycle (Figure 5), in which protein A, before becoming active and an output of the system, is converted to A* through an enzymatic reaction with a kinase K (the input of the system) and converted back through another enzymatic reaction with a phosphatase K (Klipp et al. [2005]). The basic idea is that amplification of the input K should occur through the forward cycle reaction while the negative feedback should occur through the reverse cycle reaction. In order to understand how this can be explained mathematically , we consider a simple model of the cycle, in which enzymatic reactions are modeled through one-step reaction models: $\mathrm{K} + \mathrm{A} \xrightarrow{k_1} \mathrm{K} + \mathrm{A}^*$ and $\mathrm{P} + \mathrm{A}^* \xrightarrow{k_2} \mathrm{P} + \mathrm{A}$, in which we let A_T denote the total amount of cycle protein. Along with this equations, we need to model also the binding reaction of A^* with sites in the downstream system so that, after applying singular perturbation as performed before, we obtain

$$\dot{A}^* = (k_1 A_T K(t)(1 - A^*/A_T) - k_2 P A^*) \left(\frac{1}{1+\mathcal{R}(A^*)}\right). \quad (8)$$

Comparing this equation with equation (10), we see that the gain on the input is given by $G' = k_1 A_T$, while the negative feedback gain is given by $G = k_2 P$. As a consequence, we can conclude that when A_T and P are large, the behavior of A^* should be minimally affected by retroactivity. This illustrates that phosphorylation cycles can function as insulation devices and, hence, provides another reason why these cycles are ubiquitous in natural signal transduction: they can enforce unidirectional signal propagation, which is certainly desirable in any (human-made or natural) signal transmission system. Note, however, that severe compromises may arise between retroactivity attenuation and noise amplification, which are particularly relevant in biological systems due to their intrinsic stochasticity (Jayanthi and Del Vecchio [2009]).

The hypothesis that phosphorylation cycles function as insulation devices when both the amounts of cycle protein and phosphatase are sufficiently large is appealing and can be experimentally tested. However, model (8), while providing a good intuition behind the mechanisms responsible for robustness to retroactivity, is overly simplified and hides many important details that may be relevant to the overall cycle robustness. Specifically, covalent modification cycles (phosphorylation is a special case of these) can be modeled considering the enzymatic reactions as two-step processes and can even include all of the details of the units that makeup a protein, some (or all) of which can be modified by the enzymes (Ventura et al. [2010]). In the latter case, the dynamical model of the system in the box in Figure 5 can even have 30 to 50 state variables. Hence, a mathematical framework to study insulation from retroactivity is needed that can handle models of arbitrary dimension.

In order to reach this goal, we have developed a technique to analyze and design retroactivity attenuation, which holds for arbitrarily complex biomolecular networks (Jayanthi and Del Vecchio [2011]). The basic idea exploits separation of time scales and the specific structure of interconnection between biomolecular modules, and it can be illustrated in the following simplified treatment. For system Σ in Figure 2, we seek to determine conditions under which the dynamic response of y to u is minimally affected by retroactivity s. In order to do so, we write the model of the system in its isolated configuration ($s = 0$) and in its connected configuration ($s \neq 0$) and quantify the difference between the trajectories of the isolated and connected systems. Specifically, letting u be a vector variable and assuming for simplicity that $y \in \mathbb{R}^n$ is the state of Σ, we can write the dynamics of the isolated system as

$$\dot{u} = f_0(t,u) + r(u,y),\ \dot{y} = G_1 f_1(u,y), \tag{9}$$

in which $G_1 > 0$ is a positive constant. Similarly, we can write the dynamics of the connected system as

$$\begin{aligned}\dot{\bar{u}} = f_0(t,\bar{u}) + r(\bar{u},\bar{y}), \quad \dot{\bar{y}} = G_1 f_1(\bar{u},\bar{y}) + G_2 M s(\bar{y},v)\\ \dot{v} = -G_2 N s(\bar{y},v),\end{aligned} \tag{10}$$

in which $G_2 > 0$ is also a positive constant and M and N are matrices (called stoichiometry matrices (Klipp et al. [2005]). Here, v is a vector variable that models the dynamics of the downstream system to which Σ is connected. The distinctive structure of the interconnection comes in the fact that matrices M and N are such that there is a non-singular $n \times n$ matrix B and a matrix T such that $BM - TN = 0$. This is because the entries of s physically represent the rate of reversible binding between two species and it always affects with opposite signs the species involved in the binding, one of which belongs to the upstream system and the other belongs to the downstream system. The constant G_2 models the fact that binding reactions are among the fastest reactions in biomolecular networks, so that $G_2 \gg 1$. Now, assume that we can take $G_1 \gg 1$ probably not as large as G_2 but still sufficiently larger than 1. This can be achieved, for example, by letting y be driven by protein modification reactions, such as phosphorylation or allosteric modification, which are usually much faster than protein production and decay processes (Klipp et al. [2005]). In this case, we can re-write the dynamics of the connected system (10) by using the change of variables $z = B\bar{y} + Tv$, $\epsilon_1 = 1/G_1$, and $\epsilon_2 = 1/G_2$ as

$$\begin{aligned}\dot{\bar{u}} &= f_0(t,\bar{u}) + r(\bar{u},\bar{y})\\ \epsilon_1 \dot{z} &= \quad Bf_1(\bar{u},\bar{y}),\ \epsilon_2 \dot{v} = -s(\bar{y},v),\end{aligned}$$

which is in standard singular perturbation form with two small parameters. Under the assumption that the slow manifold is locally exponentially stable (see Jayanthi and Del Vecchio [2011] for the technical conditions) the above system can be well approximated by one in which $\epsilon_1 = 0$ and $\epsilon_2 = 0$. This leads to $\bar{y} = \gamma(\bar{u})$ (the locally unique solution of $f_1(\bar{u},\bar{y}) = 0$), which is the same solution as found in the isolated system (9) when $\epsilon_1 = 0$. As a consequence, we can conclude that $\|y(t) - \bar{y}(t)\| = O(\epsilon_1)$ for $0 < t_b \leq t < T$ for some $t_b > 0$ and $T > 0$, independently of the value of G_2. This result implies that if the time scale of Σ is sufficiently faster than the time scale of the input and suitable stability conditions are satisfied, then Σ attenuates the effects of retroactivity s on the response of y to u.

In view of this result, we can consider a more realistic model of the phosphorylation cycle and exploit the natural time scale separation between phosphorylation and gene expression (controlling $K(t)$) to show retroactivity attenuation. We consider a two-step reaction model for the phosphorylation reactions, given by $\mathrm{K}+\mathrm{A} \underset{\beta_2}{\overset{\beta_1}{\rightleftharpoons}} \mathrm{C}_1 \xrightarrow{k_1} \mathrm{A}^* +$ K and $\mathrm{P} + \mathrm{A}^* \underset{\alpha_2}{\overset{\alpha_1}{\rightleftharpoons}} \mathrm{C}_2 \xrightarrow{k_2} \mathrm{A} + \mathrm{P}$ with conservation laws $P_T = P + C_2$, $A_T = A + A^* + C_1 + C_2 + C$, along with the binding of A^* with downstream sites p, that is, $\mathrm{A}^* + \mathrm{p} \underset{k_{\mathrm{off}}}{\overset{k_{\mathrm{on}}}{\rightleftharpoons}} \mathrm{C}$. The resulting differential equation model is given by

$$\begin{aligned}\dot{K} &= k(t) - \delta K - \beta_1 A_T K\left(1 - \frac{A^*}{A_T} - \frac{C_1}{A_T} - \frac{C_2}{A_T} - \frac{C}{A_T}\right)\\ &\quad +(\beta_2 + k_1)C_1\\ \dot{C}_1 &= -(\beta_2 + k_1)C_1 + \beta_1 A_T K\left(1 - \frac{A^*}{A_T} - \frac{C_1}{A_T} - \frac{C_2}{A_T}\right.\\ &\quad \left. - \frac{C}{A_T}\right)\\ \dot{C}_2 &= -(k_2 + \alpha_2)C_2 + \alpha_1 P_T A^*\left(1 - \frac{C_2}{P_T}\right)\\ \dot{A}^* &= k_1 C_1 + \alpha_2 C_2 - \alpha_1 P_T A^*\left(1 - \frac{C_2}{P_T}\right)\\ &\quad +k_{\mathrm{off}} C - k_{\mathrm{on}} A^*(p_T - C)\\ \dot{C} &= -k_{\mathrm{off}} C + k_{\mathrm{on}} A^*(p_T - C).\end{aligned} \tag{11}$$

To take this system in the form (10), we can define the gain G_1 by considering the separation of time scales between gene expression and protein phosphorylation, so that $G_1 := \frac{\beta_1 A_T}{\delta} \gg 1$, $b_2 := \frac{\beta_2}{G_1}$, $a_2 := \frac{\alpha_2}{G_1}$, $a_1 := \frac{\alpha_1 P_T}{G_1}$, and $\kappa_i := \frac{k_i}{G_1}$ for $i = 1, 2$. Similarly, we can define the gain G_2 by considering the separation of time scales between gene expression and binding reactions, so that $G_2 := \frac{k_{\mathrm{off}}}{\delta} \gg 1$ and $K_d := \frac{k_{\mathrm{off}}}{k_{\mathrm{on}}}$. By using the change of variables $z = K + C_1$ and ensuring that $A_T \gg p_T$ so that $C/A_T \ll 1$, we can re-write the system as

$$\begin{aligned}\dot{z} &= k(t) - \delta(z - C_1)\\ \dot{C}_1 &= G_1\left[-(b_2 + \kappa_1)C_1 + \delta(z - C_1)\left(1 - \frac{A^*}{A_T} - \frac{C_1}{A_T}\right.\right.\\ &\quad \left.\left. - \frac{C_2}{A_T}\right)\right]\\ \dot{C}_2 &= G_1\left[-(\kappa_2 + a_2)C_2 + a_1 A^*\left(1 - \frac{C_2}{P_T}\right)\right]\\ \dot{A}^* &= G_1\left[\kappa_1 C_1 + a_2 C_2 - a_1 A^*\left(1 - \frac{C_2}{P_T}\right)\right]\\ &\quad +G_2\left[\delta C - \frac{\delta}{K_d} A^*(p_T - C)\right]\\ \dot{C} &= -G_2\left[\delta C - \frac{\delta}{K_d} A^*(p_T - C)\right]\end{aligned}$$

which is in the form of system (10) with $u = z$, $y = (C_1, C_2, A^*)'$, $v = C$, $s = k_{\mathrm{off}} C - k_{\mathrm{on}} A^*(p_T - C)$, $M = (0,0,1)'$, $N = 1$, and $r = 0$. At this point, the main result applies with $B = \mathbb{I}$ and $T = (0,0,1)'$, so that as G_1 increases, the response of A^* to K becomes insensitive to retroactivity s. This fact is illustrated by the simulation results of Figure 6. Note that while increasing A_T contributes to attenuating the retroactivity to the output, it has the down-side of increasing the retroactivity to the input of the cycle, leading to a design tradeoff (Rivera-Ortiz and Del Vecchio [2013]). This technique is applicable to large models and was used for designing the experiments on a covalent modification cycle *in vitro* (Jiang et al. [2011]) and for designing a buffer between an

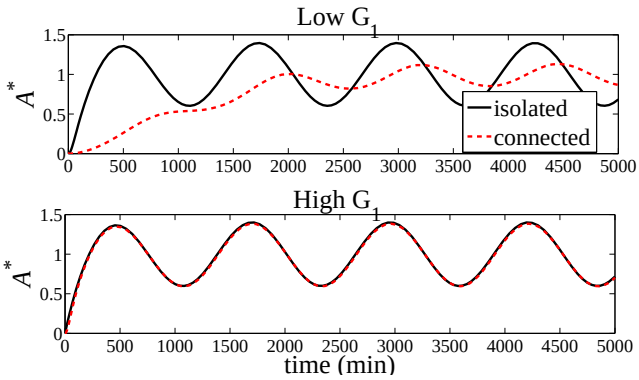

Fig. 6. Simulation of the phosphorylation cycle in (11) with low gain G_1 and high gain G_1 when $K(t)$ is a periodic signal. Specifically, we have $\delta = 0.01$, $A_T = 5000$, $P_T = 5000$, $\alpha_1 = \beta_1 = 2 \times 10^{-6} G_1$, and $\alpha_2 = \beta_2 = k_1 = k_2 = 0.01 G_1$, in which $G_1 = 10$ (upper panel), and $G_1 = 1000$ (lower panel). The downstream system parameters are $k_{\text{on}} = 100$, $k_{\text{off}} = 100$ and, thus, $G_2 = 10000$. Simulations for the connected system ($s \neq 0$) correspond to $p_T = 100$ while simulations for the isolated system ($s = 0$) correspond to $p_T = 0$

in vitro biomolecular oscillator and a load (Franco et al. [2011]). In natural signal transduction systems, phosphorylation cycles often appear in cascades. It has been shown in (Ossareh et al. [2011]) that the length of a cascade contributes to insulation from retroactivity, highlighting another reason why cycles are found in cascades.

5. CONCLUSIONS AND DISCUSSION

In this review paper, we have illustrated how problems of loading are found in biomolecular systems just like they are found in many engineering systems. Loading effects, called retroactivity, alter the behavior of a module upon interconnection and hinder modular composition of networks. Differently from electrical circuits, which can be analyzed to a large extent through linear systems theory, biomolecular network models are highly nonlinear and hence their study requires nonlinear systems theory. We have illustrated how using singular perturbation theory we can analyze and quantify retroactivity effects by obtaining equivalent system representations, just like Thevenin's theorem does for electrical circuits. We have illustrated how the design of systems that are robust to retroactivity, called insulation devices, can be formulated as a disturbance attenuation problem. This problem can be solved by exploiting the structure of the interconnection found in biomolecular networks and employing singular perturbation theory after a suitable change of variables. Insulation devices can be used in synthetic biology applications to connect systems to each other, while keeping the isolated system behavior. Experimental studies have validated this design approach.

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