Molecular Titration Promotes Oscillations and Bistability in Minimal Network Models with Monomeric Regulators

Christian Cuba Samaniego, Giulia Giordano, Jongmin Kim, Franco Blanchini, and Elisa Franco

Abstract: Molecular titration is emerging as an important biochemical interaction mechanism within synthetic devices built with nucleic acids and the CRISPR/Cas system. We show that molecular titration in the context of feedback circuits is a suitable mechanism to enhance the emergence of oscillations and bistable behaviors. We consider biomolecular modules that can be inhibited or activated by input monomeric regulators; the regulators compete with constitutive titrating species to determine the activity of their target. By tuning the titration rate and the concentration of titrating species, it is possible to modulate the delay and convergence speed of the transient response, and the steepness and dead zone of the stationary response of the modules. These phenomena favor the occurrence of oscillations when modules are interconnected to create a negative feedback loop; bistability is favored in a positive feedback interconnection. Numerical simulations are supported by mathematical analysis showing that the capacity of the closed loop systems to exhibit oscillations or bistability is structural.

Keywords: titration, oscillations, bistability, monomeric regulator, delays, synthetic biology, RNA

The construction of complex dynamic circuits from programmable parts, with behaviors that are predictable and easy to engineer, has been one of the overarching goals of synthetic biology since its early steps. The pool of available parts has been largely expanded with the development of RNA-based devices, such as riboregulators and siRNA, which are becoming increasingly popular due to the rapid programmability of RNA secondary structure and function. Recently, the CRISPR/Cas system (which relies on guide RNA molecules) has revolutionized existing methods for synthetic control of gene expression and promises to enable the creation of virtually arbitrary regulatory interactions. These rapidly evolving RNA nanotechnologies present a unique challenge in the context of building complex dynamic circuits: RNA-based regulatory interactions are noncooperative in general, meaning that one copy of RNA-driven regulator binds to its target site, rather than forming multimers like many transcription factors. Cooperativity is well-known for yielding sharp dose responses with tunable thresholds, which are nonlinearities generally required (within a feedback circuit) to obtain oscillations and multistationarity. An alternative to cooperativity is the mechanism of molecular titration, present in many natural examples of protein sequestration in signaling pathways, which can generate tunable ultrasensitive responses using stoichiometric interactions between monomers. Titration can also generate delays, which are known to promote oscillations. Thus, molecular titration is an ideal candidate mechanism to build dynamic circuits using monomeric regulators.

In this article we show that biomolecular processes driven by monomeric regulators can be combined to obtain oscillations and bistability. We consider minimal systems whose output can be repressed or activated by an increase in input monomers. These monomers bind to and control the production rate of a target molecule that represents the module output; the monomer input can be titrated by competing species that serve as constitutive activators or inhibitors. By modulating the parameters of the titration process we can control the steady state and the temporal response of the modules. In particular, we show that the total concentration of titrating species modulates the steady-state response threshold (or “dead-zone”) and the delay in the temporal response; the titration reaction rate influences the steepness of the on/off transitions in the steady state and transient response.

We interconnect modules to form canonical signal generators in biomolecular systems. Oscillators are important in biological organisms because they drive and synchronize the activity of downstream pathways. Timing signals are needed for synthetic molecular systems as well, and many artificial oscillators have been built in vivo and in vitro. Bistable systems are equally relevant as they achieve robust on–
off behaviors and serve as memory elements in signal transduction and developmental networks, as well as being important components in artificial systems.

Our approach combines numerical simulations and rigorous mathematical analysis. The models we consider have many parameters, and it is desirable to establish what their admissible dynamic behaviors are in a wide range of parameter variability, or—ideally—for arbitrary parameter choices. We employ control and dynamical systems methods and identify stability and monotonicity properties of the inhibited and activated modules; we say that these properties are structural because they do not depend on the specific parameters chosen. When these modules are interconnected to form a negative or a positive feedback loop, we can conclude that they can exclusively admit instability of oscillatory or bistable nature (respectively). These results are consistent with the famous Thomas’ conjectures. Simulations are required to identify parameter values yielding the desired dynamics: we numerically integrate the models of the candidate dynamic networks and study the parameter range in which the desired behavior is achieved. The combination of theoretical analysis and simulations reveals that direct titration is not necessary to achieve oscillations and bistability, but it significantly promotes their occurrence.

Our analysis points out design principles that render molecular titration amenable to building a variety of feedback circuits using monomeric regulators. For instance, gene networks can be regulated via protein titration, as pioneered by Buchler and colleagues (Figure 1A). Our results are particularly relevant to nucleic acid systems, because nucleic acids are naturally amenable to designing competitive binding and titration reactions to elicit nonlinear responses. Several synthetic in vitro circuits including bistable circuits and oscillators were constructed utilizing molecular titration reactions (Figure 1B). Indeed, natural examples of titration-based RNA regulatory parts abound, including sequestration of mRNA by sRNA to regulate translation and mRNA degradation (Figure 1C). These features can contribute to robust multiple target gene regulation and threshold responses. Synthetic biological circuits in the future will benefit from the targeting specificity and broad utility of CRISPR/Cas system. Nuclease mutant Cas9 proved to be useful in constructing synthetic logic circuits by utilizing simple design change of guide RNA sequences to target desired DNA.

Figure 1. Examples of natural and synthetic gene expression regulatory pathways where monomeric molecules determine the activity of a target (indirect titration, blue reaction arrows) and can be annihilated by competing species (direct titration, red reaction arrows). (A) Monomeric transcription factor titrated by an inhibitor. (B) Synthetic DNA activators binding to the promoter region of linear templates can be titrated out by complementary DNA or RNA inhibitors. (C) mRNA and ribosome binding can be prevented by small RNA molecules which titrate the mRNA. (D) Guide RNA and Cas9 bind to form the active Cas9-sgRNA complex; sgRNA is titrated by an anti-sgRNA molecule forming an inactive complex.
sequence domains for repression and activation. Still, lack of ultrasensitive response proved challenging when layering gates.\textsuperscript{9,10} It is likely that an analogous approach utilizing molecular titration can yield ultrasensitivity in CRISPR/Cas system and allow fine-tuning of circuit dynamics (Figure 1D).

**RESULTS**

**Minimal inhibited and activated modules.** We consider molecular modules where monomeric activators and inhibitors compete to determine the fraction of a target that is in an active ($X_T$) or inactive ($X_T^*$) state. We say that our models are minimal because activators and repressors are monomeric, and regulatory reactions are solely uni- and bimolecular. In the rest of this article we indicate a chemical species with an uppercase letter, and its concentration with the corresponding lowercase letter (e.g., species $A$ has concentration $a$).

An inhibited module is composed of the target $X_T$, a constitutive activator $X_A$, and is regulated by the inhibiting species $R_I$ (Figure 2A). Similarly, an activated module is composed of the target $X_T$, a constitutive inhibitor $X_I$, and an activator $R_A$ (Figure 2F). We assume that the total concentration of target is constant: $x_T + x_T^* = x_T^{\text{tot}}$ at any point in time. This assumption is reasonable if the target is, for instance, a gene whose copy number is constant. Similarly, we assume that the total concentration of constitutive inhibitor and constitutive activator are constant in each module; in other words, we assume that the time scale of their production and degradation is much slower than the regulatory reactions within the modules, and can thus be neglected for the purpose of our analysis. Like the target $X_T$, the constitutive inhibitor $X_I$ and activator $X_A$ switch between a functional and inert state while their total concentrations remain constant. This assumption is handy because it allows us to examine the behavior of each system treating the total concentration of constitutive activator/inhibitor species as a design parameter. The inert constitutive activators/inhibitors are converted back to their

![Figure 2](https://example.com/figure2.png)
active form at a constant rate. Finally, we assume that the activators and inhibitors $R_A$ and $R_I$ are produced by “input” species $U_A$ and $U_I$ respectively, and are degraded at a constant rate.

The regulators $R_A$ and $R_I$ bind directly to their target $X_T$ and $X_T^*$ controlling the amount of its active fraction. In addition, regulators can bind to the constitutive inhibitor or activator; these interactions result in titration of the regulators available for direct target binding. We will focus on the inhibited regulator in more detail. Inhibitor $R_I$ binds to active target $X_T$ ($X_T^* \cdot X_T$ complex), converting it to inactive target $X_T^*$ and releasing inert constitutive activator $X_A^*$ ($X_T^* \cdot R_I$ complex); $R_I$ is sequestered in this inactive complex; $X_T^*$ spontaneously recovers its activity over time through degradation of $R_I$ in the complex. $R_A$ also binds to free $X_A$, yielding the inert complex $X_A^* \cdot R_A$ complex where $R_A$ is sequestered. A large amount of free $X_A$ in solution can thus titrate $R_I$, delaying inhibition of the target. $R_I$ is also degraded at a constant rate. The activated module works in a similar manner.

The reactions modeling the release of $X_T$ and $X_T^*$ as inert species, and their subsequent recovery, are consistent with our assumption that the total concentration of $X_T^*/X_T$ remains constant (molecules of $X_T^*/X_T$ are not produced nor degraded, they only switch between active and inert form). In addition, these reactions model very well the toehold-mediated branch migration processes in nucleic acid transcriptional circuits. In this case, $R_I$ is an RNA species displacing a portion of the promoter $X_A$ from a synthetic gene $X_T^*$; $X_A$ displaced from $X_T$ ($X_T^* \cdot X_A$ complex) by $R_I$ forms an inert complex $X_T^*$ ($X_A \cdot R_I$ complex); RNase H degradation of $R_I$ bound to $X_A$ results in recovery of $X_A$. In general, $X_A$ and $X_T$ could be proteins or RNA species designed to bind to and titrate the input regulators $R_A$ and $R_I$ respectively. We refer to these reactions as “direct titration” or simply titration. The reactions between regulators $R_A$ and $R_I$ and the complexes $X_T^*$ and $X_T$ indicate as “inhibition” and “activation” reactions respectively, can be classified as titration reactions as well, but in this context we refer to them as “indirect titration”. Molecular titration is a well-known mechanism to generate ultrasensitivity and delays (Figure 1A); we will describe how these properties can be achieved and tuned by controlling the direct titration reaction.

The reactions defining each module are listed below. (For simplicity we denote reaction rates with the same symbols when they have the same function in the two modules.)

Inhibited module

\[
\begin{align*}
R_A & \quad \sim \quad U_A + X_T^* \quad \rightarrow \quad R_A + X_T^* \\
R_I & \quad \sim \quad U_I + X_T \quad \rightarrow \quad R_I + X_T \\
X_T & \quad \sim \quad R_A + R_I \quad \rightarrow \quad X_T \\
X_T^* & \quad \sim \quad R_A + R_I \quad \rightarrow \quad X_T^* \\
X_T & \quad \sim \quad X_T^* \quad \rightarrow \quad X_T \\
X_T^* & \quad \sim \quad R_I \quad \rightarrow \quad X_T^* \\
X_T & \quad \sim \quad R_I \quad \rightarrow \quad X_T \\
\end{align*}
\]

Activated module

\[
\begin{align*}
X_T & \quad \sim \quad U_A + X_T^* \quad \rightarrow \quad X_T + X_T^* \\
X_T^* & \quad \sim \quad U_I + X_T \quad \rightarrow \quad X_T^* + X_T \\
X_T & \quad \sim \quad X_T^* + X_T \quad \rightarrow \quad X_T + X_T^* \\
X_T & \quad \sim \quad X_T^* \quad \rightarrow \quad X_T \\
X_T^* & \quad \sim \quad X_T \quad \rightarrow \quad X_T^* \\
\end{align*}
\]

Because the total concentration of species $X_T$, $X_T^*$, and $X_A$ is constant, we can write the following mass conservation equalities: $x_T^\text{tot} = x_T + x_T^*$, $x_A^\text{tot} = x_A + x_A^*$, and $x_I^\text{tot} = x_I + x_I^*$. Figure 2A and F show a graphical representation of the two modules and their reactions. Using the law of mass action and the mass conservation equalities, we obtain the following model for the inhibited module:

\[
\begin{align*}
\dot{x}_T &= \alpha (x_T^\text{tot} - x_T)x_A - \delta x_T R_I \\
\dot{x}_A &= \kappa (x_A^\text{tot} - x_A - x_T^\text{tot}) - \alpha (x_T^\text{tot} - x_T)x_A - \frac{\nu x_A R_I}{1 + \phi} \\
\dot{R}_I &= \beta u_I - \phi R_I - \delta x_T R_I - \frac{\nu x_A R_I}{1 + \phi}. \\
\end{align*}
\]

The differential equations describing the activated module are

\[
\begin{align*}
\dot{x}_T &= \alpha (x_T^\text{tot} - x_T^*) R_A - \delta x_T x_I \\
\dot{x}_I &= \kappa (x_I^\text{tot} - x_I - (x_T^\text{tot} - x_T^*)) - \delta x_T x_I - \frac{\nu x_I R_A}{1 + \phi} \\
\dot{R}_A &= \beta u_A - \phi R_A - \alpha (x_T^\text{tot} - x_T^*) R_A - \frac{\nu x_I R_A}{1 + \phi}.
\end{align*}
\]

Boxes highlight terms associated with the titration reactions between constitutive activators/inhibitors and the input regulators. Before exploring numerically the behavior of these differential equations, we point out some important properties of the stationary and transient behavior of these systems.

Structural properties. The concentration of each species in the modules is bounded for arbitrary (positive and bounded) values of the binding rates, and of initial and total concentration of species. By assumption, the total concentration of the target $X_T$ and the total concentration of the constitutive activator $X_A$ and inhibitor $X_I$ are constant, thus $x_T^\text{tot}(t) \leq x_T^\text{tot}$, $x_I^\text{tot}(t) \leq x_I^\text{tot}$, and $x_T(t) \leq x_T^\text{tot}$ at any time. As for the regulator inputs, the presence of a first order degradation rate ensures that their concentrations are bounded. For the inhibited subsystem, for instance, note that $r_I(t) \leq \beta u^\text{max} - \delta r_I$. By applying the comparison principle, we conclude that $r_I(t) \leq r_I(0) e^{-\delta t} + u^\text{max} \beta (1 - e^{-\delta t}) / \phi$, which ensures $r_I(t) \leq \max[r_I(0), u^\text{max} \beta / \phi]$ at any point in time; see Propositions 3 and 9, Supporting Information (SI) Section 1.

Expressions that relate the steady state of the active target concentration $x_T$ to the concentration of the species producing regulators are derived in Section 1 of the SI. In the absence of titration reactions it can be shown analytically that the steady state curves that relate the input regulator source concentrations to the active target concentration are monotonic curves: the steady state mapping of the inhibited module $x_T = g(u_I)$ is decreasing; the steady state mapping of the activated module $x_T = k(u_A)$ is increasing (Propositions 1 and 7, SI, Section 1). Due to the monotonicity of the equilibrium curves, equilibria are unique once $u_I$ or $u_A$ are fixed. In the presence of titration reactions, numerical simulations indicate that monotonicity is preserved in the parameter range we considered (see Figure 2B, C, G, H). Monotonicity of the steady state input/output relationships is important to identify admissible equilibria in interconnections of modules, which will be considered in the following sections.

The behavior of these systems around the equilibrium point can be examined by linearizing the differential equations. In the absence of titration reactions, the Jacobian matrices of the inhibited and activated modules show that both systems are monotone. This property is satisfied if the total concentration of constitutive activator $x_A^\text{tot}$ and inhibitor $x_I^\text{tot}$ is larger than the concentration of target $x_T^\text{tot}$; it is also required that the difference $(x_T^\text{tot} - x_T^*)$ be larger than ratio of the recovery to activation rate $\kappa / \delta$; similarly we require that $(x_T^\text{tot} - x_T^*) \geq \kappa / \delta$. If these assumptions are satisfied, then the Jacobian of each module is a sign definite Metzler matrix, which means that both modules are input/output monotone for arbitrary choices of the remaining parameters (details are provided in the SI, Section 324).
considering these modules in the context of larger circuits.31,41 Dynamics, and stability are all important properties when equilibrium input/output maps, monotonicity of the linearized increasing their total concentrations.

In the presence of titration reactions, boundedness and stability are all important properties when considering these modules in the context of larger circuits.31,41 The fact that these properties hold for (near) arbitrary choices of parameters indicates that our minimal systems may be treated as input/output modules. These modules can be interconnected creating predictable, robust feedback loops whose net positive or negative sign does not depend on the parameters.

**The concentration of titrating species modulates the dose response threshold and the delay of the time response.** Using the parameters reported in Table 1, we numerically solved the differential equations describing the inhibited and the activated modules, examining their steady state (Figure 2B, C, G, H) and their transient response (Figure 2D, E, I, and J). The steady state fraction of active target $x_A / x^\text{tot}$ shows a Hill-type dose response to the concentration of species $U_I$ or $U_A$ that produce the regulator; the response threshold can be increased by increasing the total concentration of constitutive activator or inhibitor (titrating species). For example, Figure 2B shows that the steady state fraction of active target decreases as the concentration of $U_I$ (the species producing inhibitor) increases; as the concentration of constitutive activator (titrating species) is increased up to 500 nM, the inhibition threshold moves to the right reaching about 100 nM. A similar behavior is observed for the activated module in Figure 2G. The dynamical effect of an increase in titrating species concentration is a temporal delay in reaching steady state, as shown in Figure 2D and I; for the reaction rates chosen in this example, the delay can reach 25–30 min. We numerically explored the role of the titration reaction rate, whereby constitutive activators and inhibitors sequester available regulator input. While the titration reactions are not required to obtain the qualitative threshold-dependent dose response and the time delay, their presence sharpens both responses. As shown in Figure 2C and H, the larger the titration rate the sharper is the transition between on and off states at steady state, once a certain $U_I$ or $U_A$ input threshold is reached. Figure 2E and J show that the temporal switch between fully on and fully off states of the target becomes sharper as the titration rate increases. We remark that in the absence of titration ($\nu = 0$) the systems still exhibit a dose response threshold and a delay in the time response; large values of $\nu$ yield sharper nonlinear behaviors, in particular increased ultrasensitivity and faster temporal switch in activity. Parameter sensitivities for the inhibited and activated modules are further explored in Figures S1 and S2.

To characterize the dynamic response of the modules we integrated the differential equations varying the signal source, target and titrating species concentration. As a measure of the delay, we then quantified the rise time of the target response, defined as the time it takes for the target concentration to reach 60% of its steady state value. Results are shown in Figure 3: the rise time is most dramatically influenced by the concentration of titrating species, and increases proportionally to it; as expected, the rise time is reduced by increasing the concentration of source signal.

**Table 1. Parameters for the Inhibited System (Eqs 1–3) and for the Activated System (Eqs 4–6)**

<table>
<thead>
<tr>
<th>Rate</th>
<th>Description</th>
<th>Value</th>
<th>Other studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$ (/M/s)</td>
<td>Activation</td>
<td>$3 \times 10^4$</td>
<td></td>
</tr>
<tr>
<td>$\delta$ (/M/s)</td>
<td>Inhibition</td>
<td>$3 \times 10^4$</td>
<td>Nucleic acids: $10^4–10^6$, refs 28, 42</td>
</tr>
<tr>
<td>$\nu$ (/M/s)</td>
<td>Titration rate</td>
<td>$3 \times 10^3$</td>
<td>Protein/Protein: $10^4–10^6$, refs 43, 44</td>
</tr>
<tr>
<td>$\beta$ (/s)</td>
<td>Production of regulator</td>
<td>$5 \times 10^{-3}$</td>
<td>RNA: $10^{-3}$ to 1, refs 45, 46</td>
</tr>
<tr>
<td>$\kappa$ (/s)</td>
<td>Recovery of titrating species</td>
<td>$1 \times 10^{-3}$</td>
<td>RNA: $10^{-3}$–$10^{-2}$, refs 46, 47</td>
</tr>
<tr>
<td>$\phi$ (/s)</td>
<td>Degradation of regulator</td>
<td>$1 \times 10^{-3}$</td>
<td>Proteins: $10^{-4}$–$10^{-3}$, ref 12</td>
</tr>
</tbody>
</table>

Figure 3. Rise time of the active fraction of target in the inhibited and activated module. A significant increase in rise time occurs for low values of the input source, and large concentration of constitutive activator/inhibitor. Below a certain concentration threshold of $u_I$ and $u_A$, the rise time is small because the target is not affected by activation and inhibition reactions.
In summary, by modulating the concentration of titrating species and the titration rate, we can control the characteristics of both the dynamic and the steady state response of each module. Specifically, we can determine the threshold and steepness of the steady state nonlinearity, and we can modulate the delay of the temporal response. These features are essential to build complex dynamical systems using the titration-based modules as components.

The feedback interconnection of inhibitor and activator modules creates a negative feedback loop and is a structural oscillator. By interconnecting the inhibited and the activated module described in the previous sections, we create a negative feedback loop circuit and explore its capacity to exhibit oscillations. A scheme of the interconnection is shown in Figure 4A. The differential equations of the oscillator are

$$\dot{z}_T = \alpha_z(z^{\text{tot}}_T - z_T)x_T - \delta_T z_T z_I$$  \hspace{1cm} (7)

$$\dot{x}_T = \alpha_x(x^{\text{tot}}_T - x_T)x_T - \delta_T x_T x_T$$ \hspace{1cm} (8)

$$\dot{x}_A = \beta_x(x^{\text{tot}}_A - x_A)x_A - \delta_A x_A x_A$$ \hspace{1cm} (9)

$$\dot{x}_I = \kappa_x(x^{\text{tot}}_I - x_I)x_I - \delta_I x_I x_I$$ \hspace{1cm} (10)

$$\dot{x}_{R,I} = \beta_{x,I}(x_T - x_I)x_{R,I} - \delta_{x,I} x_{R,I} x_{R,I} - \phi_{x,I} z_{R,I}$$ \hspace{1cm} (11)

The equations are ordered to highlight two groups of variables: \(z_T, z_I, x_{R,A}\) and \(x_T, x_A, z_{R,I}\). The first group represents the inhibited subsystem, the second group represents the activated subsystem (Figure 4A). The complete list of reactions is reported in Section 2 of the SI. First, we establish mathematically that this system has the correct structure to oscillate, and that its only admissible transition to instability is oscillatory. Second, we characterize the circuit behavior as a function of the

Figure 4. (A) Schematic of the oscillator system built by interconnecting an inhibited and an activated module. (B) Trajectories of the target species when eqs 7–12 are integrated using nominal parameters (Table 2). (C) Trajectories in panel B overlapped with the system equilibrium equations (Section 2.2 of the SI). (D and E) Trajectories of the target species for variable concentrations of constitutive activators and inhibitors, obtained by integrating eqs 7–12 using nominal parameters (Table 2). The concentration of titrating species affects primarily the amplitude of oscillations.
concentration of titrating species (constitutive activator and inhibitor), of titration reaction rate, and production rate of regulators.

**Structural analysis.** The oscillator is designed to have a negative feedback loop, which is generally a necessary (not sufficient) condition for oscillations. However, eqs 7–12 are nonlinear ODEs with 16 parameters, which makes the system quite complex: it is reasonable to ask what dynamic behaviors are admissible when the parameter values are varied. In addition to verifying that the system can oscillate with numerical analysis (shown in the next section), it is possible to establish analytically that—depending on the chosen parameters—this model either behaves as an oscillator, or as a system with a unique, stable equilibrium point. We can exclude multiple equilibria. We can reach this conclusion following different routes.

In the absence of titration reactions, the system is the negative feedback interconnection of two stable monotone subsystems (Section 2 of the SI). In Figure 4A, the monotone subsystems are highlighted by gray boxes; the target species $x_T$ and $z_T$ generate a single negative loop between the modules (orange lines). The equilibrium conditions (derived analytically for each module) intersect in a single point for arbitrary choices of the parameters. Due to the boundedness of the solution of each subsystem, the solution of ODEs (eqs 7–12) is also bounded and we can identify a “box” in the space of concentrations where the solution is trapped. These properties (together with other mild assumptions, see the SI and ref 31 for details), imply that the only type of transition to instability admitted by this system is oscillatory: in other words, by changing one or more parameters (reaction rates or total concentrations), we can push the only equilibrium admitted by this system to become unstable, and this transition is driven by a pair of complex conjugate eigenvalues which correspond to an oscillatory solution. This is a behavior akin to the well-known Hopf bifurcation. We say that this is a strong candidate oscillator (Proposition 14, Section 2.1 of the SI), because the only admissible transition to instability is oscillatory. 31 This is a structural property, in the sense that it does not depend on the chosen system parameters.

When titration reactions are present the system is still a strong candidate oscillator, even though the inhibited and activated module lose their structural monotonicity properties (Section 2.2 of the SI). This can be demonstrated by computing explicitly the characteristic polynomial of the Jacobian matrix: because its coefficients are all positive (for any value of the parameters and equilibria), it cannot have nonnegative real roots (Proposition 16, Section 2.2 of the SI). Thus, unstable eigenvalues must be complex conjugate and this implies that transitions to instability can only be oscillatory. (This approach is also applied in the SI to the system in the absence of titration reactions.)

**Numerical analysis.** We integrate the differential eqs 7–12 numerically, and we test the capacity of the system to oscillate when certain parameters are varied. First, we randomly varied reaction rates and total concentrations of species 33,34 (Section 2.3.1 of the SI), and we used that information to identify a nominal set of parameters (Table 2) that yields oscillations within a period of roughly 1 h, as shown in Figure 4B. Equilibrium conditions intersect at a single equilibrium point, as expected based on our analytical derivations (Figure 4C).

Varying the concentration of titrating species affects both the amplitude and the frequency of oscillation (Figure 4D and E):

<table>
<thead>
<tr>
<th>Table 2. Nominal Parameters for the Oscillator</th>
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<tbody>
<tr>
<td>Rate Value</td>
</tr>
<tr>
<td>(a_i \text{/(M/s)})</td>
</tr>
<tr>
<td>(x_{i0} \text{/(M/s)})</td>
</tr>
<tr>
<td>(\nu_1 \text{/(s)})</td>
</tr>
<tr>
<td>(\beta_1 \text{/(s)})</td>
</tr>
<tr>
<td>(\phi_i \text{/(s)})</td>
</tr>
<tr>
<td>(\zeta_i \text{nM)})</td>
</tr>
<tr>
<td>(\zeta_i \text{nM)})</td>
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In particular, the higher \(\zeta_i\) and \(\zeta_i\), the larger the amplitude of oscillations. This is likely a consequence of two phenomena: the first is the temporal delay observed when the titrating species concentration increases in each module (Figure 2D, I and Figure 3A and B); the second is the steady state response threshold directly proportional to the titrating species concentration (Figure 2B, G). We explored systematically period and amplitude as a function of \(\zeta_i\) and \(\zeta_i\) in Figure 5A, for different values of the titration rate which we assumed for simplicity to be identical in both subsystems (\(\nu = \nu_i = \nu_i\)). All the other parameters are chosen as in Table 2. In this figure, we computed period and amplitude numerically from trajectories integrated for 20 h. These plots include also slowly damped oscillations; the region where oscillations are sustained (found as the area where the eigenvalues of the Jacobian are complex with positive real part) is inside the cyan contour. In the space \(\zeta_i\) and \(\zeta_i\), the region where oscillations are detected becomes larger as the titration rate is increased. This is likely caused by the fact that a large titration rate sharpens the stationary and dynamic response of each module. It is worth noting that the concentration of titrating species promotes oscillations only in a certain range, which may change depending on the nominal operating point; both excess or lack of \(\zeta_i\) and \(\zeta_i\) can cause loss of oscillations.

We also varied the total concentration of targets \(x_{i0}\) and \(z_{i0}\) and observed that the system is very sensitive to variations in the total concentration of inhibited target \(z_{i0}\), which is the species responsible for creating negative feedback; in contrast, the system is robust to variations in the total concentration of activated target \(x_{i0}\), as shown in Figure 5B.

The production rate of each regulator species is another particularly important parameter: the feedback interconnection of the two linearized subsystems is defined primarily by \(\beta_i\) and \(\beta_i\), which can be thought of as parameters that control the “loop gain” of the system. This is evident from the Jacobian matrix of the system, where two blocks are interconnected precisely by \(\beta_i\) and \(\beta_i\) (SI, Sections 2.1.2 and 2.2.2). The oscillatory region in the \(\beta_i\)-\(\beta_i\) space is also increased when the titration rate is higher.

While the period is only moderately affected by the variations we considered, the amplitude changes more significantly. A complete analysis of the oscillatory regions as a function of all the system parameters is presented in the SI, Section 2.3.2. Increasing the titration rate always expands the parameter areas where oscillations are observed (Figures S3, S4, S5 and S6).

**Mutually inhibiting modules create a positive feedback loop and a structural bistable system.** Two inhibited modules can be mutually interconnected by designing the output of one module to be the inhibitor input of the other, as sketched in Figure 6A. The differential equations are
Figure 5. Period and amplitude of the oscillations when key parameters are varied near their nominal values (Table 2), for increasing value of the titration rates. Axes are in log scale; parameters are varied between one tenth and ten times their nominal values. Oscillations (sustained or damped) occur in the gray areas; the cyan contour indicates the region of sustained oscillations (the linearized system has dominant unstable complex conjugate eigenvalues); the orange diamond indicates the nominal value of the parameters. (A) Variation of the concentration of constitutive activators and inhibitors $x_A^{\text{tot}}$ and $z_I^{\text{tot}}$. (B) Variations of total target concentrations $x_T^{\text{tot}}$ and $z_T^{\text{tot}}$. The system is robust to variations in the target molecule of the activated subsystem. (C) Variations of the production rates of regulators, which control primarily the strength of the feedback loop. In all cases, a larger titration rate expands the oscillatory regions.
As done for the oscillator, the variables have been grouped as \( z_T, z_A, z_{R,I} \) and \( x_T, x_A, x_{R,I} \) to separate the two inhibited subsystems (Figure 6A). The list of chemical reactions is reported in the SI, Section 3. In the next sections, first we establish if this system has the capacity to exhibit multistationary behaviors. Then, we explore the bistability regions as a function of various species concentrations and of the titration rate. Fast titration reaction rates always yield larger bistability regions, although this effect is less prominent than in the oscillator.

As for the oscillator circuit, the model in eqs 13–18 is quite complex: nevertheless, we can establish mathematically that for appropriate choices of parameters, the system either presents a single stable equilibrium or more than one stable equilibrium (accompanied by the emergence of unstable equilibria) where the dominant eigenvalue is real. There is no choice of the parameters that will make the system oscillate. In fact, the two inhibited modules in the absence of titration reactions are both stable, their solutions are bounded, and they are input-output monotone systems (SI, Sections 1.1 and 1.2). The two monotone modules are connected via a single positive feedback.
loop; in Figure 6A, the modules are represented by components in the gray boxes; the positive feedback loop is generated by the target species and is highlighted with the orange lines. The properties satisfied by the modules imply that their interconnection (eqs 13–18) can only undergo real transitions to instability;31 this kind of instability is related to the well-known saddle-node bifurcation. We say that this system is a strong candidate bistable system (Proposition 18 in the SI, Section 3.1.2): no matter how its reaction rates and total component concentrations are varied, the system dynamics are restricted to be either bistable or monostable.

In the presence of titration reactions, we cannot reach the same analytical conclusions without making assumptions on the region where the system equilibria fall (which depends on the specific choice of parameters). However, numerical simulations presented in the next section show that the presence of titration reactions expands the bistability region of the system significantly.

**Numerical analysis.** We identified a set of nominal parameters (Table 3) via a preliminary randomized exploration of the parameter space (SI, Section 3.3.1), where for simplicity we assumed the two subsystems have the same parameters. The trajectories of \( x_T \) and \( z_T \) obtained with the nominal parameter set are shown in Figure 6B, and their behavior in phase space is shown in Figure 6C. When we vary the concentration of the titrating species near their nominal values, we obtain bifurcation diagrams that clearly show the coexistence of three equilibria, of which two are stable and one is unstable, as shown in Figure 6D and E.

We then explored bistability trends in the region near the nominal parameters (Table 3). Bistability regions were determined by numerically finding intersections of the equilibrium conditions, and then checking the magnitude of the eigenvalues of the Jacobian matrix computed at the equilibrium (see SI, Section 3.3.2 for further details). In Figure 7 we vary the concentration of titrating species, of targets, and the regulator production rates (all other parameters are kept constant as in Table 3). First of all, we note that in the absence of titration reactions (\( \nu_z = \nu_x = 0 \)), the system becomes very sensitive to variations in the concentration of titrating species, and the region of bistability is very narrow; this limitation can be relaxed by increasing the titration rate (Figure 7A). In contrast, the concentration of target species can significantly vary without affecting bistability, and again a fast titration rate expands the bistable region. Similarly, changes in the regulator production rates (which determine the strength of the feedback loop) are tolerated in a reasonably large range, as long as the rates remain large. As in the oscillator, increasing the titration rate always broadens the bistable regime; this is verified numerically for all other parameters in the system (SI, Section 3.3.2, Figures S7, S8, S9 and S10).

**DISCUSSION AND CONCLUSIONS**

We have demonstrated that biomolecular modules regulated by monomeric inputs can be successfully interconnected to build two essential circuit components: an oscillator and a bistable switch. We considered deterministic ODE models of these modules, which are composed of a target molecule and of its constitutive regulators (activators or inhibitors); input regulators compete with the constitutive regulators, which act as titrating species for the input, to determine the active or inactive state of the target. The steady state and transient response of the target molecule concentration can be finely tuned by appropriate design of the titration rate and the concentration of the titrating species. Specifically, these parameters determine the “dead-zone” and steepness of the on/off transitions in the steady state dose response, and the speed and delay of the dynamic response, which promote the emergence of oscillations and bistability when modules are interconnected in feedback loops. One important finding is that, although direct titration reactions significantly increase the probability of the circuits to oscillate or have multiple steady states, they are not strictly necessary to provide the systems with the capacity for these complex behaviors.

Our numerical simulations are accompanied by rigorous mathematical analysis: we show that the modules and their interconnections have many important properties that do not depend on the model parameters (reaction rates and total

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**Table 3. Nominal Parameters for the Bistable System**

<table>
<thead>
<tr>
<th>Rate</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha_x = \alpha_z ) (/M/s)</td>
<td>( 3 \times 10^4 )</td>
</tr>
<tr>
<td>( \delta_z = \delta_x ) (/M/s)</td>
<td>( 3 \times 10^4 )</td>
</tr>
<tr>
<td>( \nu_x = \nu_z ) (/M/s)</td>
<td>( 3 \times 10^4 )</td>
</tr>
<tr>
<td>( \beta_x = \beta_z ) (/s)</td>
<td>0.0021</td>
</tr>
<tr>
<td>( \kappa_x = \kappa_z ) (/s)</td>
<td>( 3 \times 10^{-4} )</td>
</tr>
<tr>
<td>( \phi_x = \phi_z ) (/s)</td>
<td>( 1 \times 10^{-3} )</td>
</tr>
<tr>
<td>( z_T^{tot} = x_T^{tot} ) (nM)</td>
<td>100</td>
</tr>
<tr>
<td>( z_T^{tot} = x_T^{tot} ) (nM)</td>
<td>200</td>
</tr>
</tbody>
</table>

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**Figure 7.** Regions of bistability near the nominal parameters (Table 3) for increasing value of the titration rates; we assume the two inhibited subsystems have identical parameters for simplicity. Axes are in log scale; parameters are varied between one tenth and ten times their nominal values. (A) Variations in the concentration of constitutive activators; the bistability region is very narrow, but can be expanded by increasing the titration rate. (B) Bistability region as a function of the total target concentration. (C) Bistability region as a function of the regulator production rates \( \beta_x \) and \( \beta_z \), which control the strength of the feedback loop (Section 3 of the SI).
boundaries and the capacity for state toggling. 51 in vivo titration obtained via RNA polymerase sigma factors was used networks using this mechanism. Experimentally, molecular fi sensitive to the concentration of RNA polymerase, which in the signifi bounds, the oscillatory or bistable behavior of the systems is titrating species (DNA activators/inhibitors) within certain RNA bound to DNA. By increasing the concentration of which can be recovered by RNase H-mediated degradation of templates, generating inert activator or inhibitor complexes DNA activators) or activate (by displacing DNA inhibitors) the repressed depending on the presence of DNA activators (which 1B). In these systems, templates are constitutively activated or displacement reaction, whose speed is determined by sequence activity is switched on and o RNA outputs acting as mutual template regulators; template systems, the target molecules are DNA templates that produce dynamical system; however, the linearization of the modules ODEs shown in the SI, Sections 2 and 3. High gain in a feedback loop can generally destabilize a dynamical system; however, β and β influence other parts of the linearized dynamics as well. Thus their increase does not guarantee that the desired bifurcation will occur. While it is well known (experimentally and theoretically) that molecular titration can be tuned to generate nonlinear ultrasensitivities and delays, to our knowledge not many attempts have been made to build complex dynamic networks using this mechanism. Experimentally, molecular titration obtained via RNA polymerase sigma factors was used in vivo to build a bistable switch with tunable domain boundaries and the capacity for state toggling.21 In vitro, titration pathways very similar to those we describe here were used to build a bistable switch22 and an oscillator.23,24 In these systems, the target molecules are DNA templates that produce RNA outputs acting as mutual template regulators; template activity is switched on and off via a partial promoter displacement reaction, whose speed is determined by sequence and length of toehold domains on the nicked promoter (Figure 1B). In these systems, templates are constitutively activated or repressed depending on the presence of DNA activators (which complete the promoter) and DNA inhibitors (which displace the activators). RNA regulators either inhibit (by displacing DNA activators) or activate (by displacing DNA inhibitors) the templates, generating inert activator or inhibitor complexes which can be recovered by RNase H-mediated degradation of RNA bound to DNA. By increasing the concentration of titrating species (DNA activators/inhibitors) within certain bounds, the oscillatory or bistable behavior of the systems is significantly enhanced.24 The molecular oscillator is also highly sensitive to the concentration of RNA polymerase, which in the system determines the production rates. These experimental findings are consistent with the results of our analysis. A common feature of existing dynamic systems built using molecular titration23,24,28,51 is that the domains in parameter space where oscillations or bistability are achieved are generally narrow. Recently, this was clearly highlighted in a series of experiments where the in vitro oscillator by Kim and Winfree was encapsulated in microdroplets;32 the droplet production process causes a perturbation of the nominal operating point of the system (in particular, there is a loss of enzymes’ activity) and results in a striking dynamical diversity and often a loss of oscillations in droplets. This is consistent with our numerical analysis, and we speculate that this might be a consequence of the monomorphic nature of the regulators in the system. In a regime where stochastic effects are predominant, however, the lack of cooperativity may not be a significant limitation to achieve complex behaviors, depending on the system architecture.55,56

We expect that our analysis of molecular titration in the context of feedback systems will be useful to build circuits with new classes of monomorphic regulators such as the CRISPR-Cas system (Figure 1D). Logic circuits based on CRISPR-Cas have been recently characterized;57,58 however, feedback loops with bistable or oscillatory responses have not yet been obtained, presumably due to the difficulty in obtaining sharp nonlinear responses required for these behaviors. While building circuits with multiple, interconnected feedback loops may prove helpful,59 we speculate that titration of the guide RNA with overexpressed titrating RNA species might be an effective strategy.

**ASSOCIATED CONTENT**

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.5b00176.

Detailed information on our mathematical analysis, numerical simulations and additional references are included in the Supporting Information file. (PDF)

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The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work has been supported by the National Science Foundation through grant CMMI-1266402.

**REFERENCES**


