Co-transcriptional allosteric regulation of synthetic nucleic acid receptors

Daniela Sorrentino,[1,2] Simona Ranallo,[1] Eiji Nakamura,[2] Elisa Franco,[2,*] and Francesco Ricci,[1,*]

[1] *Department of Chemical Sciences and Technologies, University of Rome, Tor Vergata, Via della Ricerca Scientifica, 00133 Rome, Italy.*

[2] *Department of Mechanical and Aerospace Engineering and of Bioengineering, University of California at Los Angeles, 420 Westwood Plaza, Los Angeles, California 90095, United States.*

*Corresponding Authors, e-mail: efranco@seas.ucla.edu; francesco.ricci@uniroma2.it

ABSTRACT

We present a strategy to control allosterically the loading and release of molecular ligands from synthetic nucleic acid receptors using in vitro transcription. We demonstrate this by engineering three model synthetic DNA-based receptors: a triplex-forming DNA complex, an ATP-binding aptamer, and a hairpin strand, whose ability to bind their specific ligands can be tuned (activated or inhibited) in response to the binding of a specific RNA strand acting as allosteric effector. We then show that the receptor loading/release of ligands can be controlled co-transcriptionally by allosteric RNA regulators produced by rationally designed synthetic genes. This approach shows that highly programmable nucleic acid receptors can be controlled with molecular instructions provided by dynamic transcriptional systems, illustrating their promise in the context of coupling DNA nanotechnology with biological signaling. The kinetics of our allosteric sensors and their genetically generated inputs can be captured using differential equation models, corroborating the predictability of the approach used.

INTRODUCTION

Allosteric regulation is central to the control of many metabolic and signal-transduction pathways.[1,2] In this mechanism the binding of an effector molecule to a functional protein causes a distal site of the protein to undergo a conformational change that promotes (allosteric activation) or hampers (allosteric inhibition) the protein binding affinity for a specific ligand.[3,4] Through allostery, proteins can load and release a ligand in response to different stimuli.[5] For example, hemoglobin modulates its affinity for oxygen in response to a wide range of molecular effectors or environmental cues, including pH and temperature.[6,7] The capacity to control sites other than the effector binding site makes allostery one of the most intriguing and sophisticated naturally-

occurring mechanisms,[4,8,9] which was dubbed by Monod as the “second secret of life” (with the genetic code being the first one).[10,11]

Recapitulating allostery *in vitro* using man-made systems is not only crucial for gaining a better understanding of the key determinants of this mechanism, but also holds enormous potential to realize synthetic devices with novel functionalities. For example, *in vitro* synthetic systems have been demonstrated that, through allosteric control, can load or release a molecular cargo in response to a specific cue,[12–16] in particular in response to proteins,[17,18] nucleic acid strands,[19–22] and small molecules [23–25]. Artificial allosteric devices could play a major role toward the demonstration of increasingly complex synthetic biomolecular systems. Recently, cell-free technologies[26–28] have emerged as a simplified yet versatile environment for building biological networks,[29–32] making it an alternative route to cell-based approaches for a variety of applications in therapeutics,[33,34] point-of-use biosensing,[35–37] and diagnostics.[38–43] By taking advantage of synthetic nucleic acids and their programmable interactions, cell-free transcription/translation systems have led to the development of synthetic gene circuits that recapitulate *in vitro* some of the most complex naturally occurring systems like oscillators,[44–47]expression cascades,[48,49] and feedback loops.[26,50] Systematic methods to combine allostery and DNA/RNA devices encoding information (the two “secrets of life”) could majorly expand the repertoire of synthetic systems *in vitro.* A challenge toward this is posed by the fact that protein allostery hinges on the presence of a complex dynamic structure with the capacity to propagate conformational changes, which makes it difficult to redesign or embed allostery into engineered proteins.

Motivated by the above arguments, here we present a strategy for allosterically controlling the loading and release of molecular ligands from synthetic nucleic acid receptors through cell-free *in vitro* transcription. Our strategy consists in designing and building synthetic nucleic acid receptors (grey, Figure 1) whose ability to bind their specific ligand can be controlled (activated or inhibited) through a conformational change induced by the binding of an RNA allosteric strand (in Figure 1 an inhibitor) on a site distal from the ligand’s binding site. The RNA effector can be removed from the receptor by a complementary RNA strand through toehold-mediated strand displacement, or by RNA-degrading enzymes, restoring the receptor’s capacity to recruit its ligand. Finally, RNA effectors (activators or inhibitors) can be produced by artificial genes and affect the receptor state co-transcriptionally, achieving dynamic loading and release of ligands. We demonstrate our strategy with three different allosteric nucleic acid receptors that recruit other oligonucleotides or ATP as example ligands.

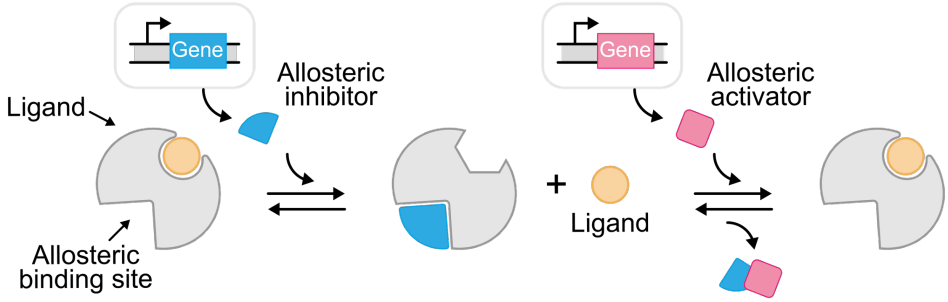

Figure 1. Co-transcriptional allosteric control of a synthetic ligand-binding receptor. *Artificial genes are rationally designed to transcribe allosteric RNA effectors (inhibitor, light blue or activator, pink) that can control the binding affinity of a DNA-based synthetic receptor (grey) for its specific ligand.*

RESULTS AND DISCUSSION

As a first model system we built a synthetic nucleic acid receptor using a triplex-forming DNA strand that is designed to bind a 9-nt DNA ligand (light blue, Figure 2) through both Watson-Crick-Franklin and Hoogsteen interactions.[51] To create a genetic mechanism to control our receptor, we first built an inhibitor gene that induces the transcription of an RNA inhibitor strand (blue, Figure 2a). The inhibitor is designed to bind to the receptor's loop portion, inducing a conformational change that triggers the release of the DNA ligand (Figure SI 1). To monitor the load and release of the ligand from the DNA-based receptor we labeled the two ends of the DNA ligand strand with a fluorophore-quencher pair. The formation of the receptor/ligand triplex complex thus results in a significant increase in fluorescence signal upon loading, followed by a decrease in signal when the ligand strand is released from the receptor.

Because the receptor's state can be regulated through an inhibitor that is produced co-transcriptionally (without any RNA purification or annealing step), by simply changing the concentration of the inhibitor gene in solution we can finely regulate the receptor's capacity to recruit its specific ligand. In our experiments we systematically titrated the inhibitor gene concentration, keeping a fixed ratio of receptor-ligand and a fixed level of T7 RNA Polymerase (T7 RNAP, 4U/μL), the enzyme that catalyzes the 5'→3' synthesis of the RNA stand from the gene. By doing so, we measured a concentration-dependent ligand release that reaches a plateau (98±2%) at a 30 nM inhibitor gene concentration (Figure 2b,c, Figure SI 2). We can also control the ligand's release from the receptor by using in the same solution different concentrations of RNase H, an enzyme that specifically degrades RNA strands in DNA-RNA heteroduplexes (Figure 2d). In this case, we observe that at higher RNase H concentrations ligand release is suppressed and is restored as the concentration of RNase H decreases (Figure 2e, Figures SI 3, 4). We achieve multiple load/release cycles with comparable efficiency through the successive addition of the inhibitor gene (in increments of 10 nM) and of RNase H (0.05 U/μL) (Figure 2f). We then introduced a second artificial gene that produces an RNA effector designed to work as an allosteric activator for the same DNA triplex-forming receptor. In this case the RNA activator co-transcriptionally displaces the RNA inhibitor by binding a single-stranded 6-nt overhang (toehold), thus restoring the affinity of the receptor for its ligand (Figure 2g). We can modulate the loading of the DNA ligand to the receptor by varying the concentration of the activator gene (0-300 nM) in the presence of a fixed concentration of inhibitor gene (1 nM) (Figure 2h, Figures SI 5, 6). Finally, the ligand's load and release from the receptor can be cycled multiple times by sequentially adding the inhibitor and activator genes to a solution containing the receptor/ligand complex (Figure 2i).

Starting from the primary chemical reactions designed to occur among ligand, receptor, genetic components, and enzymes in the system, we built an Ordinary Differential Equation (ODE) model that generally captures the trends observed in experiments (reactions and ODEs are listed in SI section SI 3). The model was fitted to the kinetic data (Figures SI 2, 4, 6), obtaining parameters that align with the literature.[52] The simulation results are shown in gray in Fig. 2c,e,h.

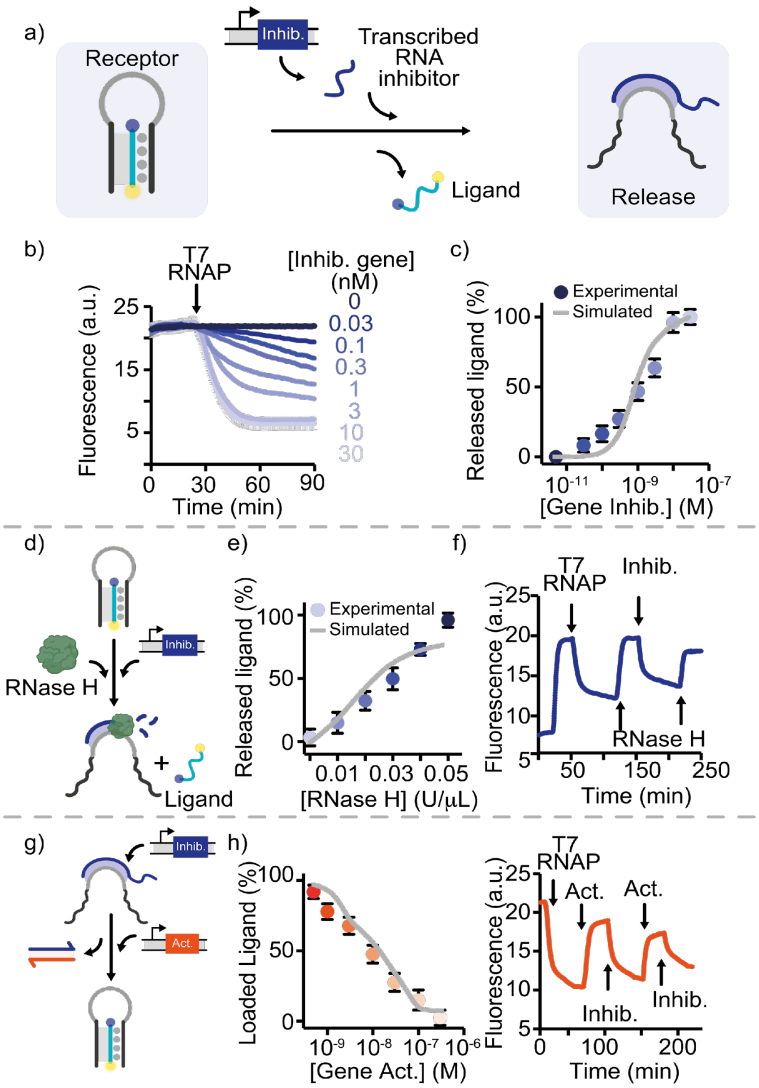

Figure 2. Co-transcriptional allosteric control of a triplex-forming DNA receptor. *a) General scheme of our DNA receptor and its RNA inhibitor. b) Time-course experiments testing the effect of varying concentrations of inhibitor gene. c) Released ligand (%) as a function of inhibitor gene concentration (end-points of traces like those shown in panel b). d) Schematic of RNase H-controlled release of the DNA ligand from the receptor. e) Released ligand (%) as a function of RNase H concentration ranging from 0.05 U/μL to 0 U/μL (time-course experiments are shown in Figure SI 3) f) Time-course experiments with successive additions of T7 RNAP (4 U/μL) and RNase H (0.05 U/μL). g) Scheme of reversible ligand loading/release. h) Loaded ligand at varying concentrations of the activator gene in the presence of a fixed concentration of inhibitor gene (1 nM) (time-course experiments are shown in Figure SI 5) i) Time-course experiments with sequential additions of inhibitor (10 nM) and activator (30 nM) genes. In the graphs, dots represent experimental values while solid grey lines represent fits (c, e, and h) obtained with the kinetic model (see SI Section SI 3.2, 3.3). All the experiments in this and the following figures were performed in 100 mM Tris-HCl, 10 mM $MgCl_2$, 150 mM NaCl at pH 7.0, 37°C. All transcription reactions were carried out, unless otherwise noted, using DNA-receptor (100 nM), DNA ligand (30 nM), T7 RNAP (4 U/μL). Solid gray lines represent fits (c, e, and h) obtained with the kinetic model (see SI Section SI 3.4, 3.5). For clarity, error bars have been shown for only one curve in these and the following figures. The error bars represent the standard deviation of measurements made on at least three independent replicates. Similar relative standard deviations (between 5% and 7%) have been obtained with the other experiments.*

Next, we developed an allosteric DNA receptor based on a ligand-binding DNA aptamer. As an illustrative example we use a well-known ATP-binding aptamer that recruits ATP through a binding-induced conformational change.[53] To achieve co-transcriptional allosteric control, we engineered the ATP-binding aptamer so that its affinity for ATP can be inhibited when a transcribed RNA strand binds to the loop domain connecting the ATP-binding portions thus leading to the release of ATP (Figures SI 7, 8). To monitor the binding events in real- time, and thus ATP loading/release, the aptamer receptor was modified at both ends with a fluorophore and quencher pair (Figure 3a). When ATP is present in solution, it binds to the receptor bringing the fluorophore and quencher into close proximity, thus resulting in a decrease of the fluorescence signal. In contrast, when the RNA inhibitor strand is transcribed and binds to the receptor, it promotes the release of ATP and leads to an increase of fluorescence. We estimate the percentage (%) of ATP released through the relative fluorescence signal change registered upon RNA transcription.

Following the same rationale described for the triplex-forming receptor, we thus designed a synthetic gene that can transcribe the RNA allosteric inhibitor and we show that ATP release can be controlled co-transcriptionally, by providing a variable amount of inhibitor gene (Figure 3b,c, Figure SI 9). Also in this case, the release of ATP from the aptamer receptor can be precisely tuned by varying concentrations of RNase H (from 0 to 0.05 U/μL) (Figure 3d,e, Figures SI 9-12), using a fixed concentration of the inhibitor gene (30 nM). Sequential addition of the inhibitor gene (10 nM) and RNase H (0.05 U/μL) allows the reversible loading and release of ATP from the receptor (Figure 3f). To provide an additional means to control the loading and release of ATP from the ATP-binding receptor, we implemented the strand displacement strategy described earlier by engineering an activator gene that produces an RNA strand acting as an allosteric activator (Figure 3g, Figure SI 13). By supplying different concentrations of the activator gene (0-100 nM) it is possible to regulate co-transcriptionally the amount of receptor-bound ATP and restore the original binding ability of the aptamer (Figure 3h, Figures SI 14, 15). An advantage of the strand displacement strategy is that it can be repeated cyclically, by sequential addition of the same amount of inhibitor and activator genes to a solution containing preformed receptor/ATP complex (Figure 3i). We finally fitted to the aptamer receptor data our general ODE model capturing the dynamics of receptor, ligand, and genetic regulators, again obtaining simulated dose-responses (shown in gray) that generally align with the data (SI section SI 3.5).

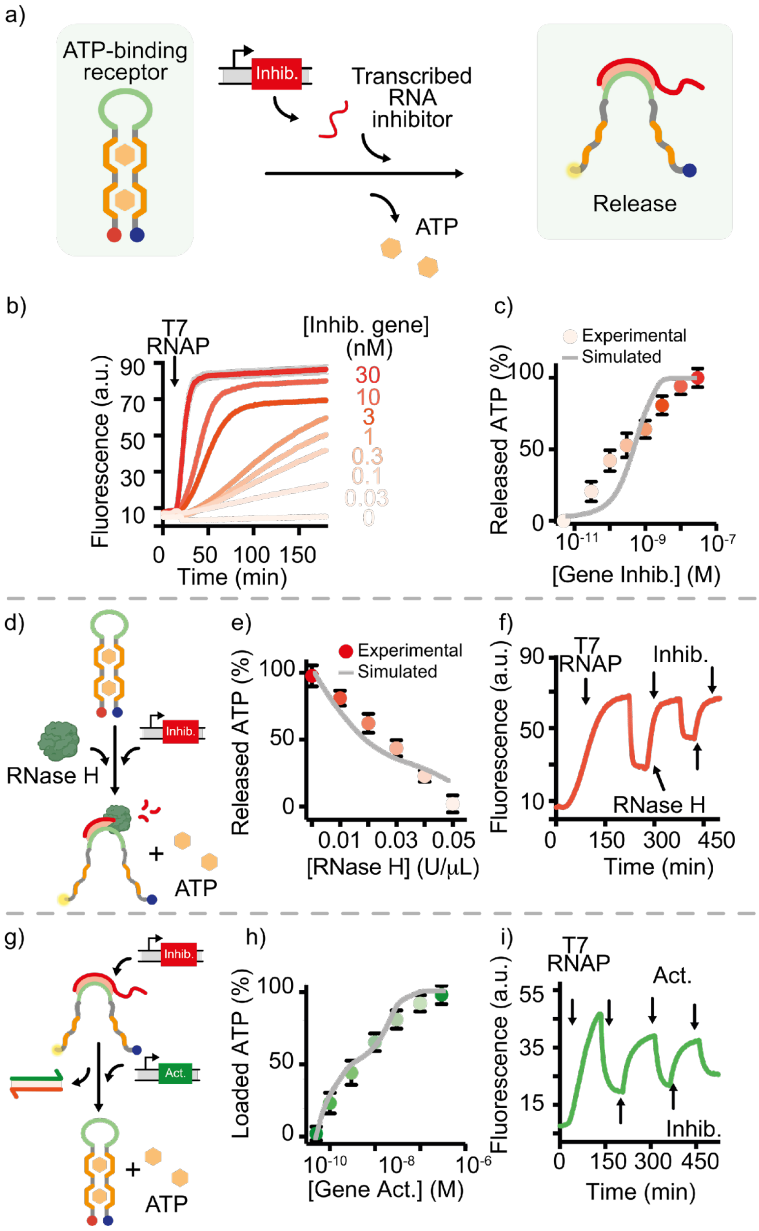

Figure 3. Co-transcriptional allosteric control of an ATP-binding aptamer. *a) Schematic of our DNA aptamer-based receptor and its allosteric RNA inhibitor. b) Time-course experiments showing the influence of varying the concentration of inhibitor gene. c) Plot showing the percentage of released ATP as a function of the gene inhibitor concentration (obtained from end points kinetic data like those shown in panel b). d) Scheme of the controlled release of the ATP in the presence of RNase H. e) Plot of the percentage of released ligand as a function of RNase H concentration (ranging from 0.05 U/μL to 0 U/μL) (time-course experiments are shown in Figure SI 11) f) Time-course experiments with sequential addition of RNA inhibitor gene (10 nM) and RNase H (0.05 U/μL) in the presence of a fixed concentration of inhibitor gene (1 nM). g) Scheme representing the reversible load/release of ATP via strand displacement. h) Plot of the percentage of loaded ATP as a function of gene activator concentration in the presence of a fixed concentration of inhibitor gene (1 nM) (time-course experiments are shown in Figure SI 14) i) Time-course experiments with sequential additions of inhibitor (10 nM) and activator (30 nM) genes at concentrations of 10 and 30 nM. All transcription reactions were carried out using ATP aptamer receptor (50 nM), ATP (1 mM), T7 RNAP (4 U/μL). In the graphs, dots represent experimental values while solid gray lines represent fits (c, e, and h) obtained with the kinetic model (see SI Section SI 3.4, 3.5).*

We finally demonstrate a third class of allosteric DNA receptors (Figure 4a). In this case the receptor is designed to have the ability to fold into a stem-loop hairpin structure that includes two tails flanking either end of the stem (orange portion, Figure 4), which act as allosteric binding sites. The ligand-binding region of the receptor is however the loop domain (grey, Figure 4), which is designed to hybridize to a single-stranded DNA ligand (purple, Figure 4), and when the receptor is bound to its ligand, the formation of the stem is hindered. The addition of an inhibitor RNA strand targeting both receptor tails promotes the hybridization of the stem domains, and induces the release of ligand (Figure SI 16). Like in previous cases, to monitor the loading and release of the DNA ligand, we labeled the hairpin with a fluorophore/quencher pair. Binding of the DNA ligand to the hairpin results in a conformational change that pushes the fluorophore away from the quencher, resulting in an increase in fluorescence.

To co-transcriptionally control the receptor, we again developed a system of RNA effectors (inhibitors and activators) that are transcribed by artificial genes. When the RNA inhibitor is produced, it stabilizes the stem-loop structure leading to the release of pre-loaded DNA ligand and to a decrease in the fluorescence signal. Like in previous cases, receptor inhibition occurs co-transcriptionally, thus we can precisely control the release of the DNA ligand from the hairpin by varying the concentration of the inhibitor gene (Figure 4b,c Figure SI 17). Also for this system, we observed a concentration-dependent decrease in ligand release through the combined use of RNase H and inhibitor gene (Figure 4d,e, Figure SI 18, 19). Our general ODE model initially failed to reproduce the kinetics of this receptor system; our fits significantly improved after including reactions that model the fact that distinct inhibitors could bind to the two branches of the receptor, leading to intermediate receptor states (SI section SI 3.6, 3.7).

Sequential additions of inhibitor gene and RNase H allow for reversible and efficient load and release of the DNA ligand from the device (Figure 4f). Also in this case, a genetically encoded allosteric RNA activator complementary to the inhibitor can be used to regulate the receptor co-transcriptionally (Figure 4g). We demonstrated precise modulation of ligand reloading by using a saturating concentration of activator gene (30 nM) and a fixed concentration of inhibitor gene (10 nM), (Figure 4h). Finally, co-transcriptional, sequential cycles of load and release of DNA ligand can be achieved by sequential addition of inhibitor and activator genes (Figure 4i).

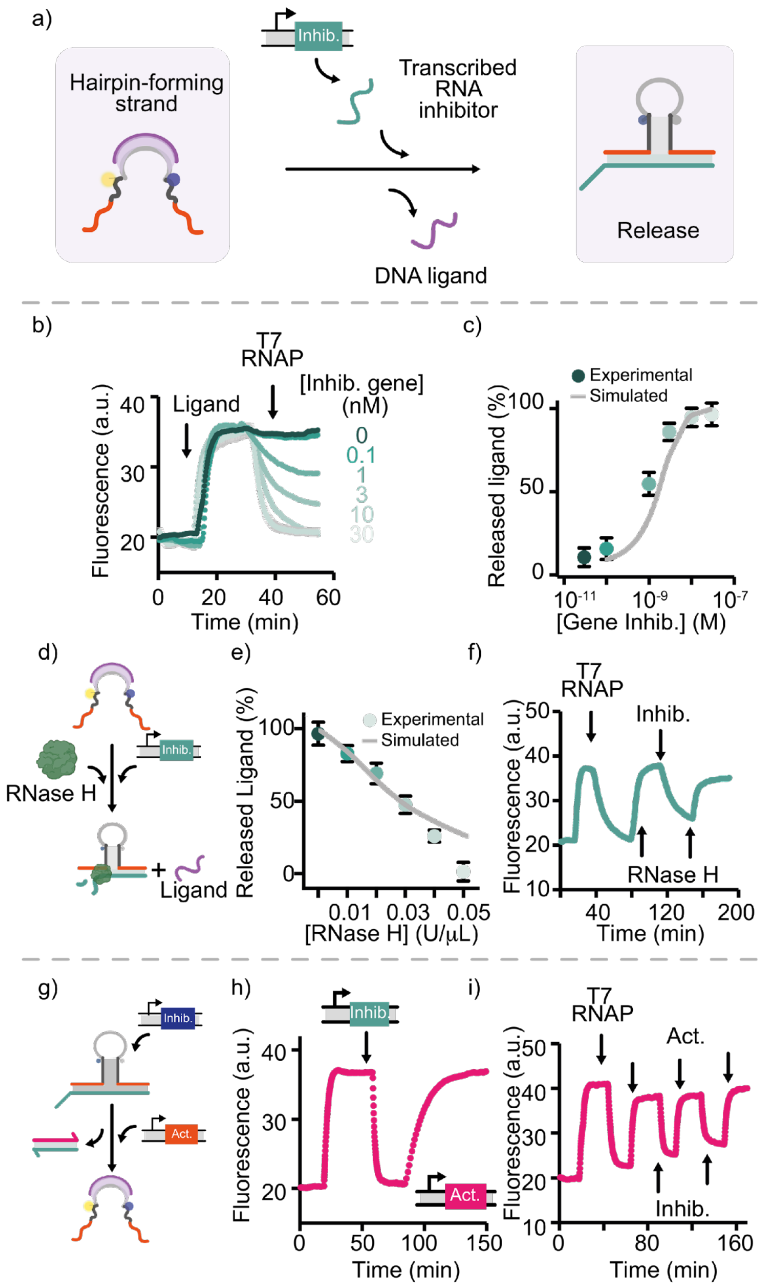

Figure 4. Co-transcriptional allosteric control of a hairpin. *a) Scheme showing the operation of the DNA hairpin-based receptor system and its allosteric RNA inhibitor. b) Time-course experiments testing different concentrations of the inhibitor gene. c) Plot of the percentage of released ligand as a function of the gene inhibitor concentration (end-points of kinetics, example shown in b). d) Scheme of the controlled release of DNA ligand in the presence of RNase H. e) Plot of the percentage of released ligand as a function of RNase H concentration (ranging from 0 U/μL to 0.05 U/μL) (time-course experiments are shown in Figure SI 18) f) Time-course experiments sequential addition of RNA inhibitor gene (10 nM) and RNase H (0.05 U/μL) in the presence of a fixed concentration of inhibitor gene (10 nM). g) Scheme of the reversible load/release of DNA target via strand displacement. h) The target loaded onto the receptor in the presence of a fixed concentration of inhibitor gene (10 nM) can be released by adding activator gene at a concentration of 30 nM. i) Time-course experiments with sequential additions of inhibitor (10 nM) and activator (30 nM) genes at concentrations of 10 and 30 nM. All transcription reactions were carried out using hairpin-forming strand (30 nM), DNA target (100 nM), T7 RNAP (4 U/μL). In the graphs, dots represent experimental values while solid gray lines represent fits (c, e) obtained with the kinetic model (see SI section SI 3.6, 3.7).*

CONCLUSION

We reported a generalizable strategy to build allosterically controlled DNA receptors, whose capacity to load/release a target ligand is regulated co-transcriptionally by synthetic genes *in vitro*. Our demonstration includes three different DNA-based receptors that were rationally designed: a triplex-forming DNA-based receptor, an ATP-binding aptamer and a hairpin. We regulated their ability to bind a specific ligand co-transcriptionally, through RNA allosteric effectors produced by the genes in which they are encoded. We demonstrated that the release of the ligand from the DNA-based receptors can be precisely tuned by varying the concentration of the inhibitor gene. By using the inhibitor gene in combination with RNase H, we suppressed the release of ligand. Finally, we showed that loading and release of the ligand is a reversible process by modulating the transcription rate of RNA inhibitors and activators, without experiencing a substantial decrease in efficiency upon successive additions of inhibitor and activator genes.

An important advantage of the allosteric nature of these receptors is that the effector sequence, which can be considered an input, is physically and chemically decoupled from the ligand, which can be considered an output. This is a sought-after property in DNA/RNA based devices, as it allows for interconnecting the operation of molecules bearing completely distinct information encoded in their sequence. Further, the molecules released by receptors may be used to regulate the production of the effectors, making it possible to build feedback loops. Our approach takes advantage of nucleic acid devices that are easy to design, build, and store, and of low-complexity cell-free conditions supporting transcription. Looking forward, our allosteric receptors may be conveniently adapted for load/release of a variety of other ligands (i.e., other RNA/DNA sequences, small molecules, and proteins), requiring simply a sequence redesign of the genetic elements producing RNA effectors. More importantly, our receptors may be coupled to transcription-based devices in more complex samples. We envision that these receptors could respond to RNA transcribed in clinically relevant contexts, and contribute to the development of new methods for biosensing, point-of-care diagnostics and drug-release devices.

ACKNOWLEDGMENT

This work was supported by the European Research Council, ERC (project n.819160) (FR), by Associazione Italiana per la Ricerca sul Cancro, AIRC (project n. 21965) (FR), by the Italian Ministry of University and Research (Project of National Interest, PRIN, 2017YER72K). EF and EN acknowledge support from NSF award CCF 2107483.

REFERENCES

(1) Nussinov, R.; Tsai, C. J. Allostery in Disease and in Drug Discovery. *Cell* **2013**, *153* (2), 293–305.

(2) Dokholyan, N. V. Controlling Allosteric Networks in Proteins. *Chem Rev* **2016**, *116* (11), 6463–6487.

(3) Nussinov, R.; Tsai, C. J. Allostery without a Conformational Change? Revisiting the Paradigm. *Curr. Opin. Struct. Biol.* **2015**, *30*, 17–24.

(4) Motlagh, H. N.; Wrabl, J. O.; Li, J.; Hilser, V. J. The Ensemble Nature of Allostery. *Nature* **2014**, *508* (7496), 331–339.

(5) Goodey, N. M.; Benkovic, S. J. Allosteric Regulation and Catalysis Emerge via a Common Route. *Nat. Chem. Biol.* **2008**, *4* (8), 474–482.

(6) Sharma, V. S.; Gilbert, G. A. Studies of Human Hemoglobin Intermediates the Double Mixing Method for Studying the Reactions of the Species Hb4(CO) and Hb4(CO)2. *J. Mol. Biol.* **1983**, *166* (4), 677–684.

(7) Bruno, S.; Bonaccio, M.; Bettati, S.; Rivetti, C.; Viappiani, C.; Abbruzzetti, S.; Mozzarelli, A. High and Low Oxygen Affinity Conformations of T State Hemoglobin. *Protein Sci.* **2001**, *10*, 2401.

(8) Bozovic, O.; Jankovic, B.; Hamm, P. Sensing the Allosteric Force. *Nat. Commun.* **2020**, *11* (1), 1–7.

(9) Wodak, S. J.; Paci, E.; Dokholyan, N. V.; Berezovsky, I. N.; Horovitz, A.; Li, J.; Hilser, V. J.; Bahar, I.; Karanicolas, J.; Stock, G.; Hamm, P.; Stote, R. H.; Eberhardt, J.; Chebaro, Y.; Dejaegere, A.; Cecchini, M.; Changeux, J. P.; Bolhuis, P. G.; Vreede, J.; Faccioli, P.; Orioli, S.; Ravasio, R.; Yan, L.; Brito, C.; Wyart, M.; Gkeka, P.; Rivalta, I.; Palermo, G.; McCammon, J. A.; Panecka-Hofman, J.; Wade, R. C.; Di Pizio, A.; Niv, M. Y.; Nussinov, R.; Tsai, C. J.; Jang, H.; Padhorny, D.; Kozakov, D.; McLeish, T. Allostery in Its Many Disguises: From Theory to Applications. *Structure* **2019**, *27* (4), 566–578.

(10) Monod, J.; Wyman, J.; Changeux, J. P. On the Nature of Allosteric Transitions: A Plausible Model. *J. Mol. Biol.* **1965**, *12* (1), 88–118.

(11) Monod, J.; Changeux, J. P.; Jacob, F. Allosteric Proteins and Cellular Control Systems. *J. Mol. Biol.* **1963**, *6* (4), 306–329.

(12) Harroun, S. G.; Prévost-Tremblay, C.; Lauzon, D.; Desrosiers, A.; Wang, X.; Pedro, L.; Vallée-Bélisle, A. Programmable DNA Switches and Their Applications. *Nanoscale* **2018**, *10* (10), 4607–4641.

(13) Chen, Y. J.; Groves, B.; Muscat, R. A.; Seelig, G. DNA Nanotechnology from the Test Tube to the Cell. *Nat. Nanotechnol.* **2015**, *10* (9), 748–760.

(14) Zhang, T.; Wei, B. Rational Design of Allosteric Nanodevices Based on DNA Triple Helix. *J. Am. Chem. Soc.* **2021**, *143* (40), 16693–16699.

(15) Mariottini, D.; Idili, A.; Ercolani, G.; Ricci, F. Thermo-Programmed Synthetic DNA-Based Receptors. *ACS Nano* **2023**, *17* (3), 1998–2006.

(16) Thirumalai, D.; Hyeon, C.; Zhuravlev, P. I.; Lorimer, G. H. Symmetry, Rigidity, and Allosteric Signaling: From Monomeric Proteins to Molecular Machines. *Chem. Rev.* **2019**, *119* (12), 6788–6821.

(17) Dagliyan, O.; Dokholyan, N. V.; Hahn, K. M. Engineering Proteins for Allosteric Control by Light or Ligands. *Nat. Protoc.* **2019**, *14* (6), 1863.

(18) Taylor, N. D.; Garruss, A. S.; Moretti, R.; Chan, S.; Arbing, M. A.; Cascio, D.; Rogers, J. K.; Isaacs, F. J.; Kosuri, S.; Baker, D.; Fields, S.; Church, G. M.; Raman, S. Engineering an Allosteric Transcription Factor to Respond to New Ligands. *Nat. Methods* **2016**, *13* (2), 177–183.

(19) Del Grosso, E.; Ponzo, I.; Ragazzon, G.; Prins, L. J.; Ricci, F. Disulfide-Linked Allosteric Modulators for Multi-Cycle Kinetic Control of DNA-Based Nanodevices. *Angew. Chem., Int. Ed.* **2020**, *59* (47), 21058–21063.

(20) Ricci, F.; Vallée-Bélisle, A.; Porchetta, A.; Plaxco, K. W. Rational Design of Allosteric Inhibitors and Activators Using the Population-Shift Model: In Vitro Validation and Application to an Artificial Biosensor. *J. Am. Chem. Soc.* **2012**, *134* (37), 15177–15180.
(21) Vinkenborg, J. L.; Karnowski, N.; Famulok, M. Aptamers for Allosteric Regulation. *Nat. Chem. Biol.* **2011**, *7* (8), 519–527.
(22) Phillips, E. A.; Silverman, A. D.; Joneja, A.; Liu, M.; Brown, C.; Carlson, P.; Coticchia, C.; Shytle, K.; Larsen, A.; Goyal, N.; Cai, V.; Huang, J.; Hickey, J. E.; Ryan, E.; Acheampong, J.; Ramesh, P.; Collins, J. J.; Blake, W. J. Detection of Viral RNAs at Ambient Temperature via Reporter Proteins Produced through the Target-Splinted Ligation of DNA Probes. *Nat. Biomed. Eng.* **2023**, 1–12.
(23) Mariottini, D.; Idili, A.; Vallée-Bélisle, A.; Plaxco, K. W.; Ricci, F. A DNA Nanodevice That Loads and Releases a Cargo with Hemoglobin-Like Allosteric Control and Cooperativity. *Nano Lett.* **2017**, *17* (5), 3225–3230.
(24) Del Grosso, E.; Idili, A.; Porchetta, A.; Ricci, F. A Modular Clamp-like Mechanism to Regulate the Activity of Nucleic-Acid Target-Responsive Nanoswitches with External Activators. *Nanoscale* **2016**, *8* (42), 18057–18061.
(25) Rodríguez-Serrano, A. F.; Hsing, I. M. Allosteric Regulation of DNA Circuits Enables Minimal and Rapid Biosensors of Small Molecules. *ACS Synth. Biol.* **2021**, *10* (2), 371–378.
(26) Karig, D. K.; Iyer, S.; Simpson, M. L.; Doktycz, M. J. Expression Optimization and Synthetic Gene Networks in Cell-Free Systems. *Nucleic Acids Res.* **2012**, *40* (8), 3763.
(27) Chappell, J.; Jensen, K.; Freemont, P. S. Validation of an Entirely in Vitro Approach for Rapid Prototyping of DNA Regulatory Elements for Synthetic Biology. *Nucleic Acids Res.* **2013**, *41* (5), 3471–3481.
(28) Garenne, D.; Haines, M. C.; Romantseva, E. F.; Freemont, P.; Strychalski, E. A.; Noireaux, V. Cell-Free Gene Expression. *Nat. Rev. Methods Primers* **2021**, *1* (1), 1–18.
(29) Silverman, A. D.; Karim, A. S.; Jewett, M. C. Cell-Free Gene Expression: An Expanded Repertoire of Applications. *Nat. Rev. Genet.* **2019**, *21* (3), 151–170.
(30) Brooks, S. M.; Alper, H. S. Applications, Challenges, and Needs for Employing Synthetic Biology beyond the Lab. *Nat. Commun.* **2021**, *12* (1), 1–16.
(31) Mazzotti, G.; Hartmann, D.; Booth, M. J. Precise, Orthogonal Remote-Control of Cell-Free Systems Using Photocaged Nucleic Acids. *J. Am. Chem. Soc.* **2023**, *145* (17), 9481–9487.
(32) Hartmann, D.; Chowdhry, R.; Smith, J. M.; Booth, M. J. Orthogonal Light-Activated DNA for Patterned Biocomputing within Synthetic Cells. *J. Am. Chem. Soc.* **2023**, *145* (17), 9471–9480.
(33) Rondon, R. E.; Groseclose, T. M.; Short, A. E.; Wilson, C. J. Transcriptional Programming Using Engineered Systems of Transcription Factors and Genetic Architectures. *Nat. Commun.* **2019**, *10* (1), 1–13.
(34) Huang, B. D.; Groseclose, T. M.; Wilson, C. J. Transcriptional Programming in a Bacteroides Consortium. *Nat. Commun.* **2022**, *13* (1), 1–13.
(35) Wu, Y.; Li, Y.; Jin, K.; Zhang, L.; Li, J.; Liu, Y.; Du, G.; Lv, X.; Chen, J.; Ledesma-Amaro, R.; Liu, L. CRISPR-DCas12a-Mediated Genetic Circuit Cascades for Multiplexed Pathway Optimization. *Nat. Chem. Biol.* **2023**, *19* (3), 367–377.
(36) Jung, J. K.; Alam, K. K.; Verosloff, M. S.; Capdevila, D. A.; Desmau, M.; Clauer, P. R.; Lee, J. W.; Nguyen, P. Q.; Pastén, P. A.; Matiasek, S. J.; Gaillard, J. F.; Giedroc, D. P.; Collins, J. J.; Lucks, J. B. Cell-Free Biosensors for Rapid Detection of Water Contaminants. *Nat. Biotechnol.* **2020**, *38* (12), 1451–1459.

(37) Pardee, K. Perspective: Solidifying the Impact of Cell-Free Synthetic Biology through Lyophilization. *Biochem. Eng. J.* **2018**, *138*, 91–97.
(38) Pardee, K.; Slomovic, S.; Nguyen, P. Q.; Lee, J. W.; Donghia, N.; Burrill, D.; Ferrante, T.; McSorley, F. R.; Furuta, Y.; Vernet, A.; Lewandowski, M.; Boddy, C. N.; Joshi, N. S.; Collins, J. J. Portable, On-Demand Biomolecular Manufacturing. *Cell* **2016**, *167* (1), 248-259.e12.
(39) McNerney, M. P.; Zhang, Y.; Steppe, P.; Silverman, A. D.; Jewett, M. C.; Styczynski, M. P. Point-of-Care Biomarker Quantification Enabled by Sample-Specific Calibration. *Sci. Adv.* **2019**, *5* (9).
(40) Takahashi, M. K.; Tan, X.; Dy, A. J.; Braff, D.; Akana, R. T.; Furuta, Y.; Donghia, N.; Ananthakrishnan, A.; Collins, J. J. A Low-Cost Paper-Based Synthetic Biology Platform for Analyzing Gut Microbiota and Host Biomarkers. *Nat. Commun.* **2018**, *9* (1), 1–12.
(41) Karlikow, M.; da Silva, S. J. R.; Guo, Y.; Cicek, S.; Krokovsky, L.; Homme, P.; Xiong, Y.; Xu, T.; Calderón-Peláez, M. A.; Camacho-Ortega, S.; Ma, D.; de Magalhães, J. J. F.; Souza, B. N. R. F.; de Albuquerque Cabral, D. G.; Jaenes, K.; Sutyrina, P.; Ferrante, T.; Benitez, A. D.; Nipaz, V.; Ponce, P.; Rackus, D. G.; Collins, J. J.; Paiva, M.; Castellanos, J. E.; Cevallos, V.; Green, A. A.; Ayres, C.; Pena, L.; Pardee, K. Field Validation of the Performance of Paper-Based Tests for the Detection of the Zika and Chikungunya Viruses in Serum Samples. *Nat. Biomed. Engin.* **2022**, *6* (3), 246–256.
(42) Woo, C. H.; Jang, S.; Shin, G.; Jung, G. Y.; Lee, J. W. Sensitive Fluorescence Detection of SARS-CoV-2 RNA in Clinical Samples via One-Pot Isothermal Ligation and Transcription. *Nat. Biomed.l Eng.* **2020**, *4* (12), 1168–1179.
(43) Lopez, R.; Wang, R.; Seelig, G. A Molecular Multi-Gene Classifier for Disease Diagnostics. *Nat. Chem.* **2018**, *10* (7), 746–754.
(44) Kim, J.; Winfree, E. Synthetic in Vitro Transcriptional Oscillators. *Mol. Syst. Biol.* **2011**, *7* (1), 465.
(45) Franco, E.; Friedrichs, E.; Kim, J.; Jungmann, R.; Murray, R.; Winfree, E.; Simmel, F. C. Timing Molecular Motion and Production with a Synthetic Transcriptional Clock. *Proc. Natl. Acad. Sci. U S A* **2011**, *108* (40), E784–E793.
(46) Semenov, S. N.; Wong, A. S. Y.; Van Der Made, R. M.; Postma, S. G. J.; Groen, J.; Van Roekel, H. W. H.; De Greef, T. F. A.; Huck, W. T. S. Rational Design of Functional and Tunable Oscillating Enzymatic Networks. *Nat. Chem.* **2015**, *7* (2), 160–165.
(47) Green, L. N.; Subramanian, H. K. K.; Mardanlou, V.; Kim, J.; Hariadi, R. F.; Franco, E. Autonomous Dynamic Control of DNA Nanostructure Self-Assembly. *Nat. Chem.* **2019**, *11* (6), 510–520.
(48) Noireaux, V.; Bar-Ziv, R.; Libchaber, A. Principles of Cell-Free Genetic Circuit Assembly. *Proc. Natl. Acad. Sci. U S A* **2003**, *100* (22), 12672–12677.
(49) Jung, J. K.; Archuleta, C. M.; Alam, K. K.; Lucks, J. B. Programming Cell-Free Biosensors with DNA Strand Displacement Circuits. *Nat. Chem. Biol.* **2022**, *18* (4), 385–393.
(50) Agrawal, D. K.; Marshall, R.; Noireaux, V.; Sontag, E. D. In Vitro Implementation of Robust Gene Regulation in a Synthetic Biomolecular Integral Controller. *Nat. Commun. 2019 10:1* **2019**, *10* (1), 1–12.
(51) Porchetta, A.; Idili, A.; Vallée-Bélisle, A.; Ricci, F. General Strategy to Introduce PH-Induced Allostery in DNA-Based Receptors to Achieve Controlled Release of Ligands. *Nano Lett.* **2015**, *15* (7), 4467–4471.
(52) Weitz, M.; Kim, J.; Kapsner, K.; Winfree, E.; Franco, E.; Simmel, F. C. Diversity in the Dynamical Behaviour of a Compartmentalized Programmable Biochemical Oscillator. *Nat. Chem.* **2014**, *6* (4), 295–302.

(53) Huizenga, D. E.; Szostak, J. W. A DNA Aptamer That Binds Adenosine and ATP. *Biochem.* **1995**, *34* (2), 656–665.