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COMMUNICATION

# Switchable and orthogonal gene expression control inside artificial cells by synthetic riboswitches

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**Here we report two novel synthetic riboswitches that respond to ASP2905 and theophylline and function in reconstituted cell-free protein synthesis (CFPS) system. We encapsulated the CFPS system as well as DNA-templated encoding reporter genes regulated by these orthogonal riboswitches inside liposomes, and achieved switchable and orthogonal control over gene expression by external stimulation with the cognate ligands.**

Artificial cells encapsulating a cell-free protein synthesis (CFPS) system[1] are a versatile platform used in various fields, such as bioreactor development,[2] biosensing,[3] directed evolution of proteins,[4] origin of life studies,[5] and the construction of therapeutic molecular robots.[6] Temporal control over gene expression offers a key technology in further imparting artificial cells with desired phenotypes and functions. Transcriptional regulation is the most popular mechanism to control gene expression inside artificial cells,[7] (and also *in vivo*), which is regulated by chemical and/or physical external stimuli.[8] Also several photo-switchable tools[9] and RNA degradation systems[10] have been developed for gene expression control in the CFPS systems. In addition to these, translational control with synthetic riboswitches[11] offers a solution to achieve switchable gene expression especially in the reconstituted CFPS systems lacking an RNA degradation system (i.e., PURE system[12]). To the best of our knowledge, there are only four cases utilizing synthetic riboswitches for translational control inside artificial cells made of phospholipid bilayer vesicles (liposomes),[7a, 13] one of which has been reported by our group. On the other hand, there are no reports on temporal and orthogonal control over gene expression inside liposomes using synthetic riboswitches. Note that the temporal and orthogonal control has been achieved at the transcriptional level.[9b,14] Temporal and orthogonal gene expression are important in precisely controlling the fate of artificial cells. In this work, we report two orthogonal riboswitches that function in the PURE system and demonstrate switchable and independent control over gene expression inside liposomes using cognate ligands.

To date, theophylline-, cyclic guanosine monophosphate- (cGMP), thiamine pyrophosphate- (TPP), and histamine-responsive riboswitches have been reported as riboswitches that function in a reconstituted CFPS systems.[11c] Among them, theophylline- and histamine-responsive riboswitches have been utilized for translational control of gene expression inside liposomes.[7a,13] In addition to these, a fluoride-responsive riboswitch was recently utilized in the development of a liposome-based biosensor,[15] although the mechanism of the riboswitch is not translational control but transcriptional control. To expand the repertoire of cell-free riboswitches, we sought to develop novel cell-free riboswitches. Recently, we reported an RNA aptamer AC17-4 that binds to ASP2905 and its derivative ASP7967.[16] Originally ASP2905 was identified as a small molecule inhibitor of potassium channel Kv12.2 in mammalian cells,[17] and we repurposed it as a membrane-permeable ligand for mammalian riboswitches.[16] We expected ASP2905 to exhibit permeability across the liposome lipid bilayer and decided to utilize the AC17-4 RNA aptamer to develop new riboswitches.

Based on the design strategy used for the development of histamine-responsive cell-free riboswitches,[13a] we designed ASP2905-responsive riboswitch variants[‡] by varying the base stem length of the AC17-4 RNA aptamer (Fig. S1, ESI†) and examined the switching activity using the PURE system (PURE*frex* 1.0) in the presence (10 μM) or absence of ASP2905

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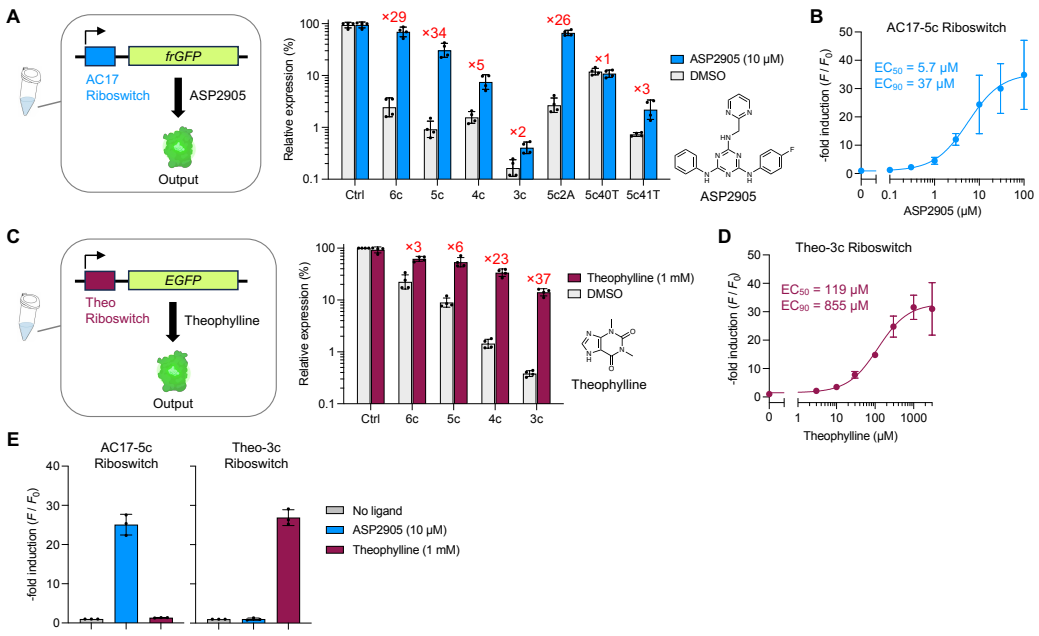


**Figure 1.** Prototyping cell-free riboswitches which respond to (A) ASP2905 or (C) theophylline. Ctrl: control without any riboswitch. 6c, 5c, 4c, and 3c stand for variations of P1 stem length of aptamer domain in riboswitch (see, Fig. S1 and S2, ESI†). The bar graphs show the mean of two independent experiments ($n$ = 4) with error bars representing the geometric standard deviation. Relative expression levels (%) of reporter green fluorescent protein (GFP) are calculated by normalization with the no riboswitch control in the absence of ligand. Numbers (Red) above the bars indicate the ON/OFF ratio. Dose dependent response of (B) AC17-5c and (D) Theo-3c riboswitches. The plots are the mean of three independent experiments ($n$ = 3) with error bars representing the standard deviation. The solid lines indicate a non-linear curve fitting, and the R-squared values were 0.8562 and 0.9305 for AC17-5c and Theo-3c, respectively. (E) Examination of orthogonality between ASP2905- and theophylline-responsive riboswitches (AC17-5c and Theo-3c variants).

(Fig. 1A; 6c, 5c, 4c, and 3c). The AC17-5c riboswitch exhibited an ON/OFF ratio of 34, which was the best performance among the four tested variants (the stem length in the aptamer domain was shortened more in the order of 6c, 5c, 4c, and 3c). The reporter (GFP: green fluorescent protein) expression level of the AC17-5c riboswitch at 10 μM ligand concentration was approximately 30% compared to that of the control without any riboswitch. We further designed AC17-5c variants (5c2A, 5c40T, and 5c41T) with a point mutation in the stem (Fig. S1, ESI†); however, these variants did not exhibit a greater ON/OFF ratio compared to the parental AC17-5c (Fig. 1A). Next, we tested the dose-dependent response of the AC17-5c riboswitch, and the median effect concentration ($EC_{50}$) was estimated to be 5.7 μM from non-linear curve fitting (Fig. 1B). In the same strategy, we redesigned theophylline-responsive riboswitches (Fig. S2, ESI†) by utilizing the theophylline-binding RNA aptamer,[18] as the previous riboswitch showed only an ON/OFF ratios of 6-15 in the PURE system.[11c] We tested four riboswitch variants with different base stem lengths of the theophylline RNA aptamer, and the Theo-3c riboswitch exhibited the best activation, of 37-fold in the presence of 1 mM theophylline (Fig. 1C). The reporter (GFP) expression level of the Theo-3c riboswitch at 1 mM ligand concentration was approximately 15% of that of the control without any riboswitch. We speculate that the stability of base stem of aptamer (Fig. S2, ESI†) negatively affected the binding affinity for the ligand, thus decreased expression level was observed (Fig. 1C), while we do not have direct evidence. We also tested the dose-dependent response of the Theo-3c riboswitch, and the $EC_{50}$ value was estimated to be 119 μM (Fig. 1D). Finally, cross-reactivity of the two riboswitch-ligand pairs was tested. At the tested ligand concentrations, no cross-reactivity was observed (Fig. 1E), and the riboswitches were activated only by the cognate ligands, indicating that these two synthetic riboswitches are orthogonal riboswitch-ligand pairs.

To control the expression of reporter genes inside liposomes using the cognate ligands, we chose folding reporter green fluorescent protein (frGFP)[19] and red fluorescent protein mScarlet-I3[20] as the reporter genes for the AC17-5c and Theo-3c riboswitches, respectively. Before conducting the liposome-based assay, we tested the riboswitch-regulated reporter gene expressions *in vitro*. At varying concentrations of ligand, reporter protein syntheses were monitored in a test tube in real time. Fluorescence from AC17-5c riboswitch-regulated frGFP was observed within 10 min after initiation of the CFPS reaction (Fig. S3A, ESI†), whereas that from Theo-3c riboswitch-regulated mScarlet-I3 was observed after 30 min (Fig. S3B, ESI†), suggesting that protein folding and chromophore maturation of mScarlet-I3 are slower than those of frGFP. This was confirmed by directly measuring the folding maturation rate in the reconstituted CFPS (Fig. S4, ESI†). In brief, we found that the maturation time of mScarlet-I3 to be 3-4-fold slower than that of frGFP, at least in the PURE system that lacks all chaperons.



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We then prepared liposomes of egg phosphatidylcholine (Egg-PC) with 20 wt % cholesterol by the water-in-oil (W/O) emulsion/transfer method,[21] and encapsulated the PURE system as well as riboswitch-regulated reporter genes. At first, we addressed whether the riboswitches could function inside liposomes and respond to stimulation by external ligands. Liposomes were incubated at 37 °C, and protein expression was analyzed by flow cytometry (FCM) at the indicated timepoints (Fig. S5, ESI†). Expression of AC17-5c riboswitch-regulated frGFP reporter was observed in response to external ligand stimulation (20 µM ASP2905), giving an ON/OFF ratio of 19 at the 180 min timepoint (Fig. 2A; No ligand vs. ON). Next, we temporarily switched OFF the AC17-5c riboswitch function by removal of ligand from the external solution (see, Materials and Methods, ESI†) at the 30 min timepoint, and again switched the riboswitch ON at the 90 min timepoint. As expected, frGFP fluorescence increased during 0-30 min and 90-180 min, and almost no fluorescence intensity change was observed during 30-90 min (Fig. 2A; ON-OFF-ON), supporting that gene expression was switchable. On the other hand, it should be noted that protein expression level inside liposomes cannot be decreased to a complete OFF state ($t$ = 0) without coupling with the protein degradation system or secretion system. Similarly, gene expression control of the Theo-3c riboswitch-regulated mScarlet-I3 reporter was examined. In the presence of 2 mM theophylline in the liposome external solution, gene expression was upregulated 25-fold at the 180 min timepoint (Fig. 2B; No ligand vs. ON). We then tested the temporal control of gene expression as with the AC17-5c riboswitch, and found that mScarlet-I3 expression was not observed during 0-30 min but increased during 30-60 min, terminated during 60-90 min, and increased again after 90 min (Fig. 2B; ON-OFF-ON). Responses to the presence and absence of theophylline were seen to be delayed. We attribute these observations to slow chromophore maturation of mScarlet-I3 (Fig. S3B and S4C, ESI†), and this hypothesis was supported by the similar fluorescence increase observed in the positive control liposomes (Fig. 2B; ON, 0-30 min). Nevertheless, we confirmed that mScarlet-I3 expression is switchable.

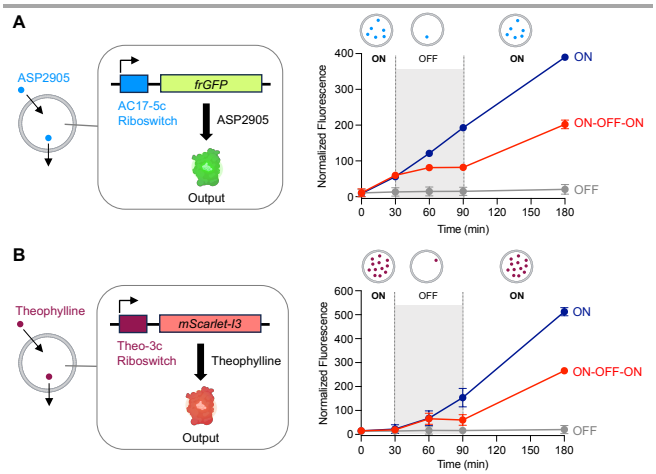


**Figure 2.** Temporal control over gene expression of (A) AC17-5c riboswitch-regulated frGFP and (B) Theo-3c riboswitch-regulated mScarlet-I3 inside liposomes. The plots are the geometric mean of two independent experiments ($n$ = 4) with error bars representing the geometric standard deviation. 10,000 particles were analyzed twice by flow cytometry in each experiment. Fluorescence from frGFP or mScarlet-I3 was normalized by co-encapsulated Alexa Fluor 647-conjugated ovalbumin, which correlates linearly with the volume of each liposome.

As shown in the bulk reaction mixture, AC17-5c and Theo-3c riboswitches do not cross-react with each other (Fig. 1E), thus we co-encapsulated these orthogonal riboswitches into liposomes to demonstrate switchable and orthogonal control of gene expression (Fig. 3A). At first, expression of AC17-5c riboswitch-regulated frGFP was induced by AS2905 for 30 min. Next, the liposome external buffer was exchanged to theophylline-containing but ASP2905-free buffer, and liposomes incubated for 60 min to induce the expression of Theo-3c riboswitch-regulated mScarlet-I3, while terminating the frGFP expression. Finally, the buffer was again exchanged to ASP2905-containing theophylline-free buffer and incubated for 120 min to restart the frGFP expression but terminating that of mScarlet-I3. As expected, FCM analyses showed that frGFP was synthesized in the first step, while no expression of mScarlet-I3 was observed (Fig. 3A; 0-30 min). In the second step, we confirmed the expression of mScarlet-I3, with almost no additional expression of frGFP (Fig. 3A; 30-90 min). In the third step, frGFP expression was recovered (Fig. 3A, 90-210 min). Due to the delayed maturation time, mScarlet-I3 fluorescence increased for the first 60 min in the third step, however no additional fluorescence increase was observed in the latter half.

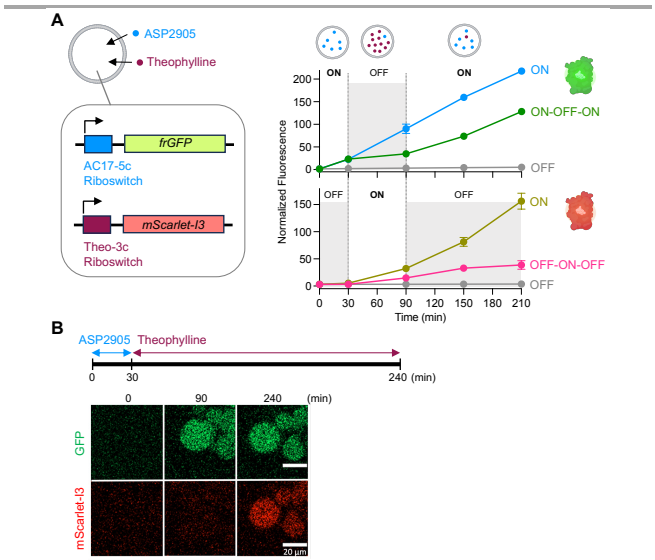


**Figure 3.** (A) Temporal and orthogonal control of gene expression with external stimulations by cognate ligands. Upper panel: normalized frGFP fluorescence. Lower panel: normalized mScarlet-I3 fluorescence. The plots are the geometric mean of two independent experiments ($n$ = 4) with error bars representing the geometric standard deviation. 10,000 particles were analyzed by flow cytometry in each experiment. Fluorescence from frGFP or mScarlet-I3 was normalized by co-encapsulated Alexa Fluor 647-conjugated ovalbumin, which correlates linearly with the volume of each liposome. (B) Fluorescence microscopy observation of reporter protein expressions in liposomes. Green and red panels indicate the expression of AC17-5c riboswitch-regulated frGFP and Theo-3c riboswitch-regulated mScarlet-I3, respectively.



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We also observed the control of orthogonal gene expression inside liposomes using confocal fluorescence microscopy. We imaged liposomes on a BSA-coated glass plate (see, Materials and Methods, ESI†) and carried out real-time observation on the induced switching of gene expression by exchanging the extravesicular buffer (Fig. 3B). Firstly, liposomes were incubated in ASP2905-containing and theophylline-free buffer for 30 min, and then the buffer was exchanged to theophylline-containing and ASP2905-free buffer. After 90 min, frGFP expression was clearly observed in liposomes, while that of Scarlet-I3 fluorescence was not. After 240 min, frGFP fluorescence hardly changed but mScarlet-I3 fluorescence was clearly observed. Switchable and orthogonal gene expression were therefore confirmed by microscopy.

In summary, we have developed two novel synthetic riboswitches, AC17-5c and Theo-3c, based on existing RNA aptamers, and demonstrated the orthogonality of the riboswitches in the reconstituted CFPS system (Fig. 1). Using these cell-free riboswitches with translational control mechanisms, we have demonstrated a switchable gene expression inside liposomes encapsulating the reconstituted CFPS system, by externally adding or removing membrane-permeable ligands (Fig. 2). Furthermore, switchable and orthogonal gene expression control inside liposomes were possible (Fig. 3). This control system can be used for sequential protein expression in artificial cells, for example by first expressing Sec translocase which folds and translocates membrane proteins, followed by membrane proteins of interest, to create artificial cells with diverse functionality. We envision that orthogonal riboswitches will be useful to control the fate of the artificial cells in future biomedical research; on one hand the production of therapeutic peptide/protein is induced, but on the other hand self-destruct is induced when the cells become unnecessary.

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## Conflicts of interest

There are no conflicts to declare.

## Notes and references

‡ Optimization of the 5' untranslated region after the aptamer sequence was carried out in the development of protein-responsive riboswitches (data not shown). We will publish the contents in the future separately to this paper.


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