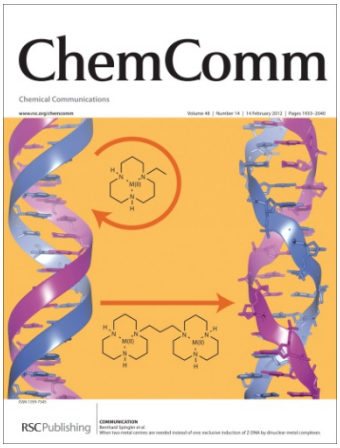

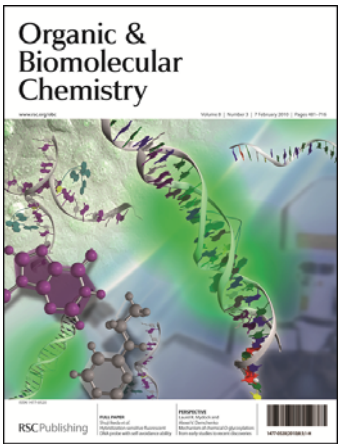

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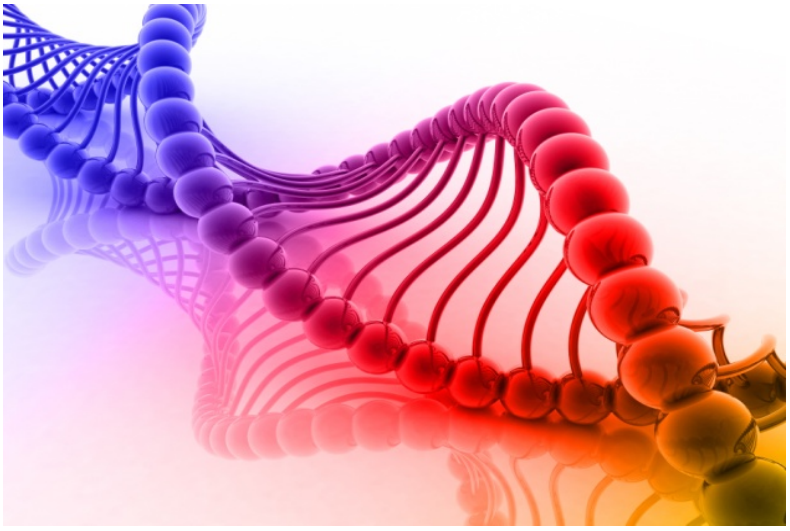

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RNA-templated molecule release induced protein expression in bacterial cells†‡

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We have developed a system to release a biologically active molecule in response to the sequence of a target gene. The releasing system, which was triggered by the reduction of an azidomethyl group, was successfully applied to protein expression induced by the release of IPTG triggered by endogenous RNA in bacterial cells.

The ideal drug should function by selectively targeting cells that are disease-causing organisms, or cure diseased cells or cancerous cells.[1] A smart concept has been proposed by Taylor and Ma in 2000,[2] which involves drug release based on genetic information, in other words, nucleic acid-templated drug release. The concept offers a number of approaches including increasing drug selectivity for chemotherapy,[3] bacterial-directed activation[4] and nucleic acid sensing that involves the detection of RNA in cells or genetic diagnosis. Various chemistries have been reported. A major focus of DNA/RNA-templated reactions has been on nucleic acid sensing *in vitro* and *in vivo*. These reactions are nucleophilic ligation reactions,[5] template native chemical ligations[6] and template Staudinger reactions.[7] Some of methods have been applied to molecular releasing *in vitro*. Those are photochemical reactions,[8] hydrolysis reactions[2,9] and the template Staudinger reactions.[10] The nucleic acid dependent chemical photocatalyst successfully generated toxic singlet oxygen by continuous photo-irradiation in living cells.[11]

Here, we present a RNA-templated molecular releasing system that causes the expression of a protein in living cells. In order to develop a system to release a molecule in response to a target RNA sequence, we selected isopropyl-β-D-thiogalactoside (IPTG) as the release molecule (Fig. 1). The addition of IPTG to bacteria is a long-standing approach to induce expression of plasmid based genes for the production of recombinant proteins under the control of the *lac* operator.[12] The *lac* repressor usually binds to the *lac* operator in the plasmid vector and target protein overexpression is repressed. When the IPTG modified probe was introduced into *Escherichia coli* (*E. coli*), the templated Staudinger reaction occurs on the target RNA, followed by the elimination reaction. Next the elimination reaction proceeds to release IPTG inside the cells. Upon IPTG binding with a *lac* repressor, it activates the promoter and induces the expression of GFP. Therefore, we can evaluate the efficiency of the molecular release based on genetic information by monitoring the fluorescence signal in living cells.

We have designed and synthesized two different DNA probes that recognize different sites of the target gene (Fig. 1 and 2); one probe has an azidomethyl-caged immolative linker containing a model molecule at the 3′ terminal end, whereas the other probe has triphenylphosphine (TPP) as the reducing reagent at the 5′ terminal end. The functional molecules containing hydroxy or amino groups can be conjugated to the α-hydroxy group of the mandelate core of the immolative group by a carbonate ester or carbamate linkages, respectively, and are released from the probes triggered by the Staudinger reaction.[10]

Initially, we tested whether the probe can release the molecule by reduction of the azidomethyl group *in vitro*. In this design the probe emits a fluorescence signal upon releasing the quencher molecule

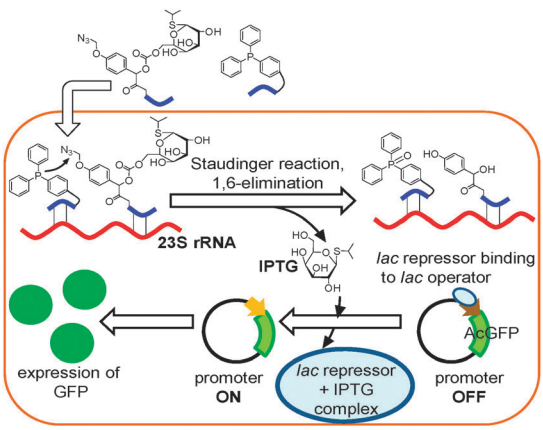

Fig. 1 IPTG release by the templated reaction induced expression of GFP.

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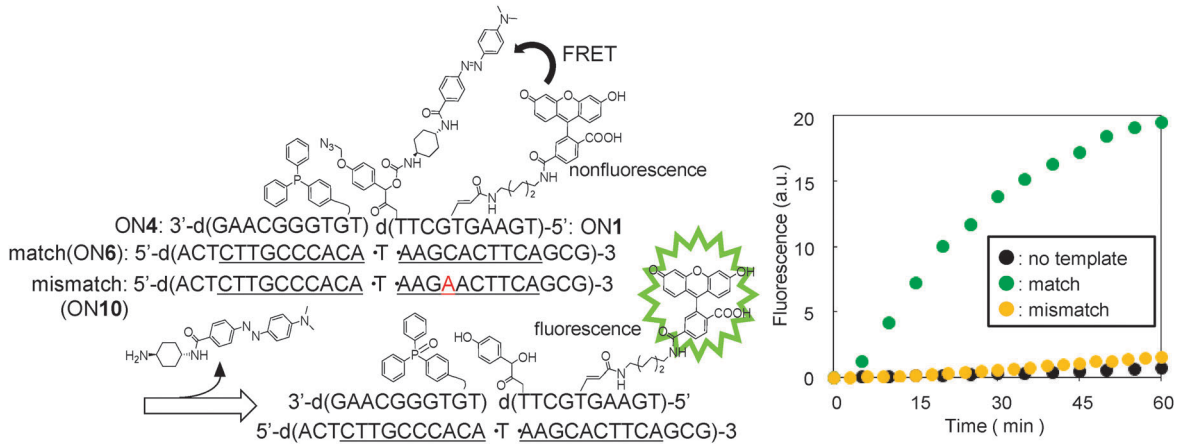

Fig. 2 Time course of the fluorescence intensity in the reaction between 50 nM ON **1** and 250 nM ON **4** with a 50 nM matched (ON **6**) or mismatched template (ON **10**) in 20 mM Tris-HCl buffer (pH 7.2) containing 100 mM $MgCl_2$ and 1 μg ml^{-1} BSA. The reaction was monitored by excitation at 490 nm and emission at 522 nm.

(Fig. 2). *p*-Methyl red was used as the quencher. The quencher and fluorophore molecules are positioned close to each other through the immolative linker on the DNA probe. The molecular releasing probe (ON **1**) has *p*-methyl red, which was connected to a caged linker and fluorescein. When *p*-methyl red was released by reduction of the azidomethyl group, efficiency of the fluorescence resonance energy transfer (FRET) decreased and the fluorescence signal was observed to increase. The synthesis of the azidomethyl-caged immolative linker **12** containing *p*-methyl red is shown in Scheme S1 (ESI‡), and the probes and target sequences are shown in Fig. 2 and Fig. S1 (ESI‡). When a high concentration of dithiothreitol was added to a solution of ON **1**, a strong fluorescence signal appeared as a result of releasing *p*-methyl red on ON **1** (Fig. S2, ESI‡). Thus, we have confirmed that the reduction of the azidomethyl group became a trigger of molecular release. We then tested whether the molecular releasing probe can release *p*-methyl red in response to the target sequence. The molecular releasing probe (ON **1**) and the TPP probe (ON **4**) incubated with or without template DNA, and the fluorescence signal at the emission wavelength of 522 nm was monitored over 60 min (Fig. 2). In the presence of the match sequence template DNA (ON **6**), the fluorescence signal increased in a time dependent manner and reached saturation at 60 min. The releasing yield at 60 min was 62%. The rate is relatively slow compared with previously reported reactions.[2,5–7,9–11] In a previous study, we reported that the reduction of an azidomethyl group on the template was completed in 3 min.[7c] Therefore, the rate-limiting step of molecular release appears to be the elimination step that follows the Staudinger reaction. Conversely, in the absence of the template, no significant increase in fluorescence was observed and the yield was 3%. In the presence of a single-base mismatched template (ON **10**), the reaction yield was only 5%. These results indicated that the probes were selective.

The rates of the DNA-templated chemical reactions were dependent on the distance between the reactive groups.[9a,13] We then examined the influence of the distance between the caged linker and reducing reagent on the efficiency of molecular release (Fig. S3, ESI‡). As expected, changing the distance between the caged linker and reducing reagent influenced the rate of molecular release. The releasing yield was 62% (n = 1), 51% (n = 2), 27% (n = 3) or 28% (n = 4) in 60 min ("n" is the number of nucleotide gaps). The increasing numbers of nucleotide gaps reduced the efficiency of molecular release. In addition, the no gapped template (n = 0) gave a yield of 23% and the releasing rate was slower than the one or two gapped template used. The immolative group was conjugated to the DNA probe by a rigid acetylene linker. So the steric hindrance between the molecular releasing and reducing probes contributed to the low releasing rate on the no gapped template. When the one nucleotide gapped template was used, the releasing rate was the largest when compared with other conditions. Thus, subsequent experiments were carried out using the one nucleotide gapped template.

Next, we tested the fluorescence detection of 23S rRNA in the *E. coli* JM109 (DE3) strain using a molecular releasing probe. Probes were designed for targeting the sequence 968–998 on the 23S rRNA, which is known to be accessible by the standard FISH probe.[14] The molecular releasing probe and the TPP probe with a matched sequence are both 15 bases long (Fig. S1, ESI‡). The TPP probe with a scrambled sequence was used as a negative control. The targeting sequence had one nucleotide gap between the molecular releasing and TPP probes. The cells were incubated in the buffer containing low concentration of SDS in the presence of the probes for 60 min at 37 °C and analyzed by flow cytometry (Fig. S4, ESI‡). The set of matched sequence probes offered significantly higher fluorescence signals than the controls. This result shows that molecule release was performed in response to the target RNA in bacterial cell.

Finally, we tried the expression of the protein based on genetic information in living cells. The synthesis of IPTG based caged linker **14** is shown in Scheme S2 (ESI‡). The 6-hydroxymethyl group of the galactosyl moiety of β-galactosides appears to be of considerable importance.[12] Substitution of the 6-hydroxy group of galactose with, for example, a methyl group destroys its induction capabilities. Therefore, caged linker **14** cannot function as an inducer. The release of IPTG from the molecular releasing probe (ON **13**, Fig. S1, ESI‡) was found by expression of GFP in *E. coli* JM109 (DE3). The cells were incubated with the buffer in the presence of the molecular releasing probes for 30 min, suspended in an LB/Amp medium and incubated for 24 h at 37 °C. After 24 h, these cells are

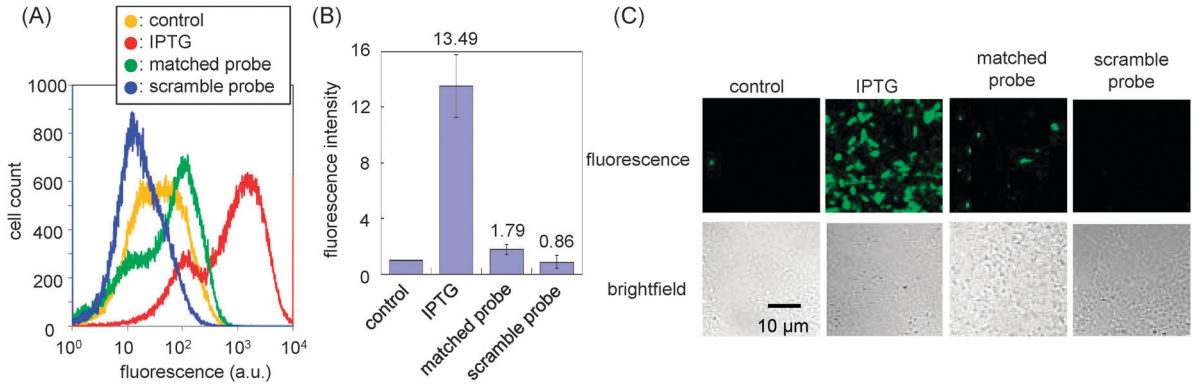

Fig. 3 Detection of the expression of GFP under the control of the *lac* operator in *E. coli* JM109 (DE3) cells. (A) Flow cytometry histogram showing cell-count frequency *vs.* fluorescence intensity for each probe. These histograms correspond to cells that were treated by no probe (control); by IPTG (positive control); by matched probes; and by scramble probes. (B) Medians of fluorescence intensity and signal-to-background ratios calculated from the flow cytometry histogram. (C) Imaging of GFP in *E. coli* JM109 (DE3) cells. Microscope settings were as follows: excitation: 488 bandpass filter; emission: 500/10 bandpass filter.

analyzed by flow cytometry and a fluorescence microscope. The flow cytometry histogram is presented in Fig. 3A and the median values of fluorescence intensity are plotted in Fig. 3B. The values on the bars indicate the signal-to-background (S/B) ratio, which is defined as the relative ratios of fluorescence intensity to the background signal from the cell (no IPTG or probes). The S/B ratios were 1.79 ± 0.35 and 0.86 ± 0.45 for matched and scrambled sequences, respectively. Fig. 3C shows the fluorescence images and bright field images of the cells. A strong fluorescent signal was observed in the case of the matched probe pair. Conversely, a negligible signal was observed from the scrambled probe pair. These results clearly showed that the expression of GFP was induced by the release of IPTG based on gene information in the cells.

In this study, we chose the 23S rRNA as a target of the templated reaction. The growing bacterial cells contain about 70 000 ribosomes[15] and the cell volumes in *E. coli* were 0.6–0.7 μm.[3,16] From these values, the concentration of 23S rRNA in the *E. coli* is calculated to be ~180 μM. The concentration of IPTG required for 50% of the operator DNA to be released from the *lac* repressor–operator complex is 2.2 μM.[17] Thus, in this model experiment, the templated reaction could produce the required amount of IPTG to induce protein expression. While rRNA represents 80–85% of intracellular RNA, mRNA represents only 1–4% of the total RNA content. Therefore, in the case of using this releasing system as target mRNA, it is necessary to select a molecule that is effective at lower concentration (order of ~nM; for example dexamethasone[18] or doxorubicin[3]).

In conclusion, we have developed a novel molecular release system based on genetic information. The system was based on the Staudinger reduction and 1,6-elimination. The molecular releasing probes were designed and synthesized for this chemistry. After reduction of the azide group to the amino group, the molecular releasing probe released the quencher or IPTG. The system was shown to detect both DNA and RNA sequences and be sensitive to a single base mismatch. We successfully applied the probe to the release of a functional molecule in response to endogenous RNA, and the released molecule induced protein expression in bacterial cells.

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