Light-activated gene expression from site-specific caged DNA with a biotinylated photolabile protection group†

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A new method for site-specific caging of plasmid DNA was developed to enable light-activation of gene expression in living cells by exposure to a low dose of light.

Spatiotemporal regulation of gene expression within living cells and organisms provides exciting possibilities for elucidating complex biological processes.[1] In particular, light-activated 'caged' nucleic acids are promising chemical tools for the spatiotemporal control of gene expression.[2] Plasmid DNAs encoding genes are generally selected for target gene regulation on the basis of their stability and ease of use. However, only a few research groups have reported caged plasmid DNAs.[2b,c] This is mainly because the gene expression of conventional caged plasmids could not be effectively induced by a low dose of light.[2b] In the conventional caging method, a random reaction between photolabile protecting groups and the phosphate backbone of DNA is employed. This caging reaction produces a number of different plasmid structures, ranging from plasmids with no bound caging groups to plasmids with a large number of caging groups. In addition, some caging groups are located on sites that are unrelated to the expression of the target gene. To block gene expression perfectly, a high average number of caging groups per plasmid is needed. Therefore, activation of gene expression required a high dose of light. However, irradiation with a high dose of light caused a decrease of gene expression and cell death because of phototoxicity.[2b,d] Accordingly, blocking gene expression with a small number of caging groups is required to enable light-activation with a low dose of light. Here, we report the design, synthesis, and photo-induced activation of a novel, caged plasmid DNA that is site-specifically labelled with one biotinylated photolabile group within the promoter region.

Site-specific caging of expression plasmids was performed by a three-step procedure. First, site-specific amino-modification was performed by overlap extension PCR using a 5′-amino-modified DNA primer (Fig. 1a).[3] Second, the amine group incorporated within the PCR product was reacted with the amine-reactive group of the biotinylated caging agent **1** (Fig. 1b). Third, streptavidin was bound to the biotin group of the site-specifically caged plasmid DNA, as a steric regulator to block transcription factors binding to the promoter sequence. In this study, the enhanced green fluorescent protein (EGFP)-expression plasmid, pEGFP-N1, was selected as a model plasmid to analyze gene expression in living cells. Site-specific caged plasmid DNAs were transfected into HeLa cells by lipofection and EGFP expression was then activated by UV light in living cells.

The primers used in the overlap extension PCR are shown in Fig. 1a. The forward primer was covalently modified with a hexylamine moiety at the 5′ end. Each primer was extended by PCR using a DNA polymerase. According to previous reports,[3] the product of overlap extension PCR is a circular molecule, containing two staggered nicks. In our design, the calculated Tm value of the 51 bp sequence between the two staggered nicks is above 65 °C. Therefore, under physiological conditions, the PCR product probably annealed to form a circular double-stranded molecule. In addition, the hexylamine moiety was located in the nick, 3 bp upstream of the TATA box of the cytomegalovirus (CMV) promoter.

Biotinylated caging agent **1** was designed based on a 6-bromo-7-hydroxycoumarin-4-ylmethyl (Bhc) group. Bhc is a recently developed photolabile protection group that has a photosensitivity superior to that of classical 2-nitrobenzyl (NB)-based

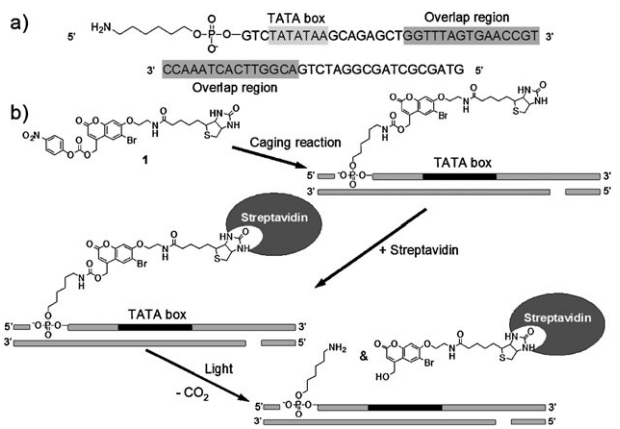

Fig. 1 Structure of overlap primers used in the overlap PCR (a) and schematic illustration of the synthesis and photochemistry of site-specific caged DNA (b).

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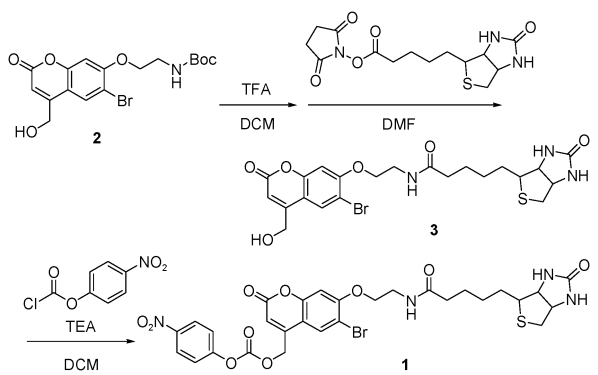
photolabile groups.[4a] Because of its high sensitivity to long-wavelength UV light, Bhc has been previously employed for preparing caged analogues of various biologically active molecules.[4] In addition, it was recently reported that the 7-hydroxyl moiety of the Bhc group can be alkylated without decreasing the overall photo-sensitivity to light.[4d] Based on this finding, we decided to attach a biotin moiety at the 7-position of Bhc (Fig. 1b). According to previous reports,[4a,d,e] the biotinylated caging agent was activated at the 4-position with 4-nitrophenyl carbonate ester as an amine-reactive group. As shown in Fig. 1b, biotinylated caging agent **1** can be covalently attached to PCR products *via* a carbamate bond.

The synthesis of **1** was accomplished as shown in Scheme 1 (see ESI† for detailed procedures). The starting compound **2** was prepared in three steps, as previously reported.[4e] The Boc protecting group of **2** was removed in dichloromethane (DCM) containing 10% trifluoroacetic acid (TFA). The amine group was then biotinylated using biotin-*N*-hydroxysuccinimidyl ester and triethylamine (TEA) in DMF to yield compound **3** (56% over two steps). Next, the biotinylated caging agent **1** was obtained by reaction of **3** with 4-nitrophenyl chloroformate and TEA in CH_2Cl_2.

The PCR product was incubated with biotinylated caging agent **1** at various concentrations of **1** from 0 to 10 mM in a sodium carbonate buffer. After removal of excess caging agent **1** by column chromatography and gel filtration, site-specific caging of PCR products was investigated by restriction enzyme digestion and electrophoretic mobility shift analysis.[5] Site-specific caged DNA was digested with restriction enzymes (*SnaB*I and *Sma*I) at two sites: 213 bp upstream (*SnaB*I site) and 98 bp downstream (*Sma*I site) of the TATA box. The resulting DNA fragments, containing a caged fragment, were incubated with fluorescent (Alexa Fluor 488)-labelled streptavidin for 1 h at room temperature, followed by electrophoresis in 1% agarose gel. By imaging the EtBr-fluorescence of the electrophoresed gel, contrary to our expectation, two DNA fragments of approximately 100 bp and 250 bp were detected in the non-caged DNA sample (*i.e.* amino-modified PCR products with 0 mM caging agent **1**) (Fig. 2a). Considering the restriction sites, it was expected that a DNA fragment of 318 bp would be obtained by digestion with the restriction enzymes (Fig. S1, ESI†). This result could be explained by the expected DNA fragment being split into two shorter DNA fragments because the DNA duplex between the two staggered nicks, which were introduced in overlap extension PCR, probably denatured during electrophoresis. Considering the sites of the two nicks (3 bp upstream and 41 bp downstream of the TATA box), the two DNA fragments were identified as the 108 bp fragment between the upstream nick and the *Sma*I site and the 261 bp fragment between the *SnaB*I site and the downstream nick (Fig. S1, ESI†). If this hypothesis is correct, only the shorter fragment has a hexylamine moiety and can react with biotinyl caging agent **1**. In fact, as the concentration of **1** increased, the band of the shorter fragment became thinner (Fig. 2a, position **A**), while the band of the longer fragment remained almost the same (Fig. 2a, position **B**), and coincidentally, a new, blurred band appeared (Fig. 2a, position **C**). In the Alexa Fluor 488-fluorescent image of the same gel, a blurred band was similarly observed at the same position as the new band in the EtBr-fluorescent image (Fig. 2b). Thus, this new band at position **C** is derived from conjugates of caged DNA and fluorescent-labelled streptavidin (Fig. 2b). When intact pEGFP-N1 containing no hexylamine moiety was incubated with caging agent **1** and analyzed as described above, the band of digested DNA did not shift as the concentration of **1** increased (Fig. S2, ESI†). These results indicate that PCR products were site-specifically modified with a biotinylated caging group through chemical reaction between the hexylamine moiety of the PCR products and the biotinylated caging agent **1**. Furthermore, it was also confirmed that streptavidin can bind to the biotinylated caging group on the DNA, as designed.

Next, we investigated the photo-cleavage of site-specific caged DNA *in vitro*. The site-specific caged DNA solutions were irradiated with UV light for various periods of time. The photo-irradiated samples were analyzed as described above. As the dose of light increased, the band of caged DNA fragment-streptavidin conjugate became thinner (Fig. 2c and d, position **C**), and simultaneously, the band of the non-caged DNA fragment thickened (Fig. 2c, position **A**). Thus, the



Scheme 1 Synthesis of the biotinylated caging agent **1**.

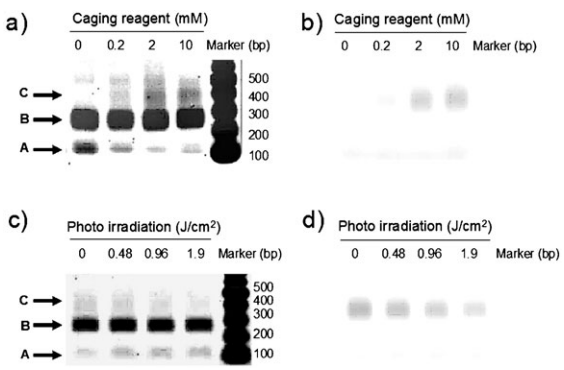


Fig. 2 Electrophoretic mobility shift analysis of the caging reaction and the photo-cleavage reaction. (a), (b) Site-specific caged plasmid DNA, prepared at various concentrations of caging agent **1** (0–10 mM), was digested with two restriction enzymes (*Sma*I and *SnaB*I) and incubated with fluorescent (Alexa Fluor 488)-labeled streptavidin, followed by electrophoresis in 1% agarose gel. (c), (d) Site-specific caged plasmid DNA prepared at the caging agent concentration of 10 mM was irradiated by ultraviolet light (365 nm, 0–1.9 J cm^{-2}) and analyzed as in (a), (b). After electrophoresis, the fluorescent images of gels were obtained by scanning the fluorescence of EtBr (a), (c) and Alexa Fluor 488 (b), (d).

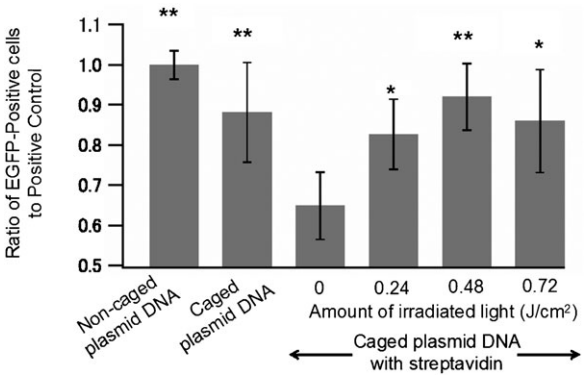


Fig. 3 Effect of light on the EGFP expression in HeLa cells transfected with site-specific caged plasmid DNA. Non-caged and site-specific caged plasmid DNA with or without streptavidin were transferred into HeLa cells by lipofection for 2 h. After transfection, some groups of HeLa cell cultures were individually exposed to various doses of 365 nm UV light (0–0.72 J cm^{-2}). After incubation for 24 h, each HeLa cell culture was analyzed by flow cytometry. The percentages of EGFP expressing cells were normalized to that of cells transfected with non-caged plasmid DNA. Each data point represents the mean ± S.E. ($n > 5$). *$p < 0.01$ and **$p < 0.001$ *versus* caged plasmid DNA with streptavidin irradiated with 0 J cm^{-2} of UV light (*t*-test).

in vitro photo-cleavage of the biotinylated caging group ('uncaging') by UV irradiation was confirmed.

Gene expression of site-specific caged plasmid DNA was investigated in living cells. The EGFP-expression level of cells transfected with non-caged plasmid DNA was 47 ± 4.1% ($n = 9$, mean ± S.E.). The percentage of EGFP-positive cells in each sample was normalized with that of this positive control sample (Fig. 3). Without streptavidin, the expression from caged plasmid DNA was slightly decreased compared with the positive control (88 ± 12%, in Fig. 3). On the other hand, streptavidin bound to the caged plasmid DNA decreased the expression level to 65 ± 8.3% (Fig. 3, caged plasmid DNA with streptavidin irradiated with 0 J cm^{-2} of UV light). This decrease due to the combination with streptavidin was statistically significant ($p < 0.001$; *t*-test on 16 degrees of freedom, Fig. 3). These results indicate that site-specific caged plasmid DNA in combination with streptavidin can suppress gene expression as predicted.

To investigate how caged plasmid DNA with streptavidin suppressed EGFP expression, we first visualized streptavidin using fluorescent (*R*-phycoerythrin)-labelled streptavidin and confocal laser scanning microscopy. When *R*-phycoerythrin-labelled streptavidin bound to caged plasmid DNA was transferred into cells, several red-fluorescent aggregates were observed in transfected cells (Fig. S3,ESI†). On the other hand, when *R*-phycoerythrin-labelled streptavidin was transferred with no plasmid DNA or non-caged plasmid DNA, no red-fluorescent aggregate was detected in cells (Fig. S3, ESI†). These results strongly suggested that streptavidin was introduced into living cells with caged plasmid DNA as expected. Next, as a control experiment, we investigated the gene expression of the plasmid DNA caged at a site that is not related to transcription: a site more than 900 bp downstream of the poly(A) sequence in pEGFP. As a result, transferred caged plasmid DNA both with and without streptavidin did not change the level of EGFP expression (98 ± 6.2% and 97 ± 6.7% of non-caged plasmid DNA, respectively, $p > 0.4$; t-test on 4 degrees of freedom). This result indicated that caging of plasmid DNA at the site related to transcription is required to suppress gene expression. Additionally, this result confirmed that binding with streptavidin did not change the transfection efficiency of caged plasmid DNA. Thus, the decrease in gene expression from plasmid DNA caged near the TATA box was probably due to streptavidin competitively blocking transcription factor binding to the TATA box.

To investigate the light-activation of site-specific caged plasmid DNA in living cells, the transfected cells were exposed to various doses of UV light. With exposure to UV light, the EGFP expression from the caged plasmid DNA with streptavidin increased to above 83% of positive control (Fig. 3). The maximum expression level was 92% at a light-dose of 0.48 J cm^{-2}. A simple calculation suggested that almost three quarters of the suppressed gene expression was activated by light-irradiation under these conditions. In a previous report of a caged pGFP randomly modified with NB-based photolabile groups,[2b] at the maximum, only one third of the suppressed gene expression was recovered by light-doses of 0.25–5.6 J cm^{-2}. Thus, the present site-specific caged plasmid DNA was more effectively activated by light.

In summary, we have developed a new method for site-specific caging of plasmid DNA with biotinylated caging agent **1**. The suppressed gene expression of site-specific caged plasmid DNA was activated by a low dose of light more effectively than that of conventional randomly-caged plasmid DNA. In principle, the present site-specific caging method may be applicable to any plasmid DNA coding gene, shRNAs and other functional RNAs, such as RNA aptamers. Thus, this site-specific caging method is a promising technology for light-controlled gene expression within living cells.

Notes and references

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