

Optical Control of Antibody Activity by Using Photocleavable Bivalent Peptide–DNA Locks

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Antibody-based molecular recognition plays a central role in today's life sciences, ranging from immunoassays to molecular imaging and antibody-based therapeutics. Control over antibody activity by using external triggers such as light could further increase the specificity of antibody-based targeting. Here we present bivalent peptide–DNA ligands containing photocleavable linkers as a noncovalent approach by which to allow photoactivation of antibody activity. Light-triggered cleavage of the 3-amino-3-(2-nitrophenyl)propionic acid peptide linker converted the high-affinity bivalent peptide–DNA lock into weakly binding monovalent ligands, effectively restoring antibody targeting of cell-surface receptors. In this work, a proof of principle was provided with an anti-hemagglutinin antibody, but the molecular design of the lock is generic and applicable to any monoclonal antibody for which an epitope or mimotope of sufficient affinity is available.

The ability to develop monoclonal antibodies with high affinities and specificities against a broad range of molecular targets has revolutionized the life sciences. Antibody-based immunoassays play a dominant role in disease diagnostics and molecular imaging, and six out of the ten bestselling drugs are antibodies or antibody derivatives.^[1] Despite their intrinsic affinities and specificities, however, antibody-based therapeutics and molecular imaging agents still suffer from background binding and toxicity through binding to receptors in non-diseased tissue.^[2–4] New molecular strategies are needed to increase the specificity of antibody-mediated targeting, either by rendering their activity conditional on the presence of other biomarkers or by allowing their local activation by external triggers.^[5–8] Protease-activatable therapeutic antibodies have been developed by fusing blocking peptides or protein domains to the antigen-binding domains through protease-cleavable linkers.^[9] Binding of these recombinantly engineered blocked antibodies can be restored by tumour-associated proteases, allowing tumour-specific antibody activation in a mouse model. Another

approach for making antibody activity dependent on the presence of specific biomarkers was reported by Church and co-workers, who used DNA origami to control antibody binding sterically, by immobilizing antibody fragments in the interior of a DNA barrel.^[10,11]

An alternative and molecularly less demanding approach to reversible control antibody activity is to use bivalent peptide–DNA conjugates in which the use of a rigid double-stranded (ds) DNA linker ensures efficient bridging of the two antigen-binding sites, yielding a stable bivalent interaction between antibody and ligand. Specific release of antibody blockage was demonstrated by triggering the disruption of the bivalent ligand into two monovalent ligands, either by the introduction of MMP-specific protease recognition sequences or by disruption of the dsDNA linker through toehold-mediated strand displacement.^[12,13]

Light is a very attractive trigger for controlling molecular interactions, because of its high spatiotemporal resolution and noninvasive nature. The above examples represent efforts to control antibody activity by using endogenous local triggers, but generic molecular strategies that allow control of antibody activity by light are mostly lacking.^[14] Self and co-workers reported the preparation of light-activatable antibodies by blocking nucleophilic amino acid side chains at the antibody exterior or with 1-(2-nitrophenyl)ethanol, with diphosgene as a coupling agent.^[15,16] Although in specific examples UV irradiation resulted in restoration of antibody binding, this blocking approach results in a modification of all nucleophilic side chains and thus yields heterogeneous mixtures of blocked antibodies. Here we report the use of bivalent peptide–DNA ligands containing photocleavable linkers as a generic and noncovalent approach to allow optical control over antibody activity (Figure 1 A).

To demonstrate this new approach we used a commercially available IgG1-type monoclonal antibody against the hemagglutinin (HA) epitope found in influenza viruses. This antibody recognizes the HA epitope YPYDVPDYA with a monovalent affinity of ≈ 1 nM.^[17] A bivalent peptide–dsDNA conjugate consisting of a 20 bp dsDNA linker with the HA peptide conjugated to the 5'-ends of both DNA strands was shown to form a very tight antibody–lock complex, showing complete inhibition at a 1:1 antibody/ligand ratio at low nM concentrations.^[12] To make antibody blockage light-sensitive we used the photolabile 3-amino-3-(2-nitrophenyl)propionic acid (Anp).^[18] This *ortho*-nitrobenzyl-containing β -amino acid is commercially available and was originally developed to allow cleavage of peptides from solid-phase resins under mild conditions. More recently, it has been successfully applied to allow optical control over peptide-based molecular processes, including pep-

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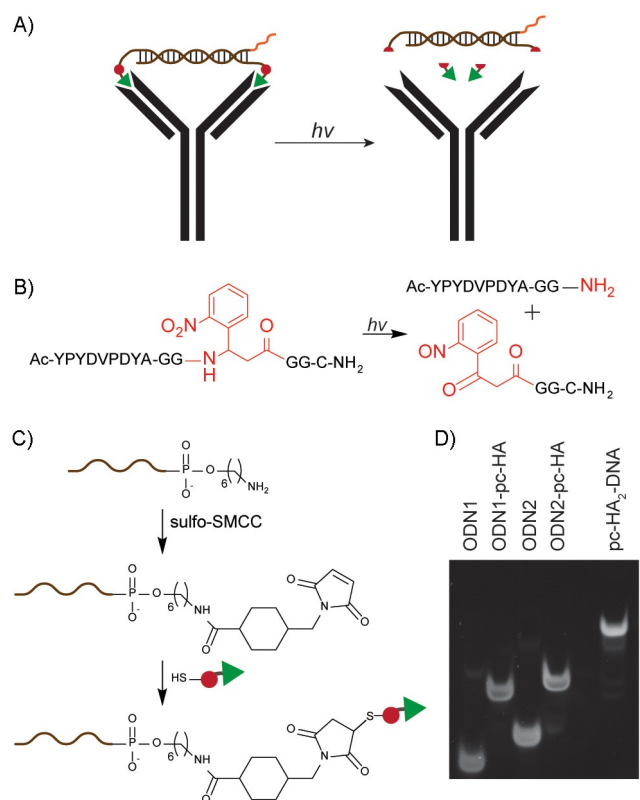


Figure 1. Development of the photocleavable peptide–DNA lock. A) Schematic representation of the mechanism of the photocleavable lock. Before illumination, the bivalent lock binds to the two antigen binding sites of the antibody. With 365 nm light the lock is cleaved, resulting in two more weakly binding monovalent peptides and one dsDNA complex. B) Photoreaction of the photocleavable Anp group incorporated at the C terminus of the HA peptide. Irradiation at 365 nm results in cleavage of the backbone. C) To obtain the peptide–oligonucleotide conjugates, a 5′-amino-functionalized oligonucleotide is first treated with the heterobifunctional sulfo-SMCC crosslinker. Subsequently, the maleimide-activated oligonucleotide is allowed to react with the C-terminal cysteine residue in the photocleavable peptide, resulting in the formation of the peptide–oligonucleotide conjugate (POC). D) A polyacrylamide gel (15%), stained for DNA with SybrGold, showing the ssDNA strands (ODN1/ODN2) and the peptide–oligonucleotide conjugates of the ssDNA strands with the photocleavable HA peptide (pcHA). Hybridization of the two complementary POCs results in complete formation of the photocleavable ligand (pc-HA₂-DNA).

peptide–protein interactions and peptide-based self-assembly.^[19–22] Illumination of Anp with 365 nm light results in cleavage of the N–C α bond, effectively cleaving the peptide into an N-terminal part with an amidated C terminus and a C-terminal part with the remainder of the Anp group attached to the N terminus (Figure 1 B).

Solid-phase peptide synthesis was used to synthesize a photocleavable HA peptide consisting of the HA epitope, a flexible linker with the Anp group flanked by two glycine residues on each side and a C-terminal cysteine unit for oligonucleotide conjugation (Ac-YPYDVPDYA-GG-Anp-GG-C-NH₂, Figure 1 B). Control experiments showed the peptide to be stable under ambient light for several hours, but also that its complete cleavage could be induced by illumination for 10 min with 365 nm light (Figure S1 in the Supporting Information). Next,

the C-terminal cysteine residue of the peptide was coupled to 5′-NH₂-functionalized oligonucleotides by using the hetero-bifunctional crosslinker sulfosuccinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (sulfo-SMCC; Figure 1 C). Complementary peptide–oligonucleotide conjugates (POCs) containing 20 and 28 bp single-stranded (ss) DNA (Table S1) were synthesized and purified by HPLC, resulting in >99% purity (Figure S2). A 20 bp dsDNA linker was chosen because previous work had shown efficient blocking of antibody activity by using linkers of either 20 or 35 bp, corresponding to 7 and 12 nm, respectively.^[12,13] Hybridization of the two complementary POCs in a 1:1 ratio yielded a bivalent photocleavable peptide–DNA lock (pc-HA₂-DNA) with a 20 bp dsDNA linker and an 8 bp toehold sequence (Figure 1 D). The toehold sequence was introduced to allow dual control over antibody activity by using either light, through photocleavage of the peptide linker, or by disruption of the DNA linker by means of a DNA-displacement reaction.

To establish the efficiency of the pc-HA₂-DNA system as a photoactivatable blocking agent, competition assays were performed with fluorescence polarization to monitor binding of the anti-HA antibody to a fluorescein-labelled HA peptide. As expected, titration of unblocked anti-HA antibody to 2 nM of fluorescently labelled HA peptide resulted in an increase in fluorescence polarization, consistent with a *K_d* value of (0.52 ± 0.06) nM (Figure 2 A). In contrast, no increase in fluorescence polarization was observed upon addition of anti-HA antibody pre-incubated with 2 equivalents of pc-HA₂-DNA, showing efficient blockage of the antibody by the bivalent peptide–dsDNA lock. Irradiation of the antibody–lock complex at 365 nm for 10 min showed that most of the antibody activity could be restored. The titration curve for the photoactivated antibody does not completely overlap with that of the free antibody. This can be explained by competition between monovalent HA peptide cleavage products and the fluorescently labelled peptide for binding to the anti-HA antibody and is confirmed by the fact that the titration curve for the photocleaved complex is very similar to that obtained with anti-HA antibody in the presence of two mole equivalents of unlabelled monovalent HA peptides. Measurement of fluorescence polarization as a function of illumination time showed antibody activation to be rapid and complete within 5 min (Figure 2 B). Parallel analysis of the photocleavage reaction by native PAGE confirmed these results, showing complete conversion of the bivalent peptide–dsDNA lock to dsDNA containing either one or none of the HA epitope peptides within 5–10 min (Figure 2 C).

Having established efficient light-triggered antibody activation *in vitro*, we also explored whether the bivalent peptide–DNA locks could be used to control antibody targeting to cells. Yeast cells displaying the HA peptide fused to the yellow fluorescent protein citrine were employed to allow quantitative monitoring of cell-surface antibody binding by flow cytometry (Figure 3 A, B).^[13] To establish the efficiency of antibody blocking, a titration experiment was performed in which the photocleavable lock was titrated to 1 nM Alexa 647-labelled anti-HA antibody. Next, HA-expressing yeast cells were added and, after an incubation period of 60 minutes, the yeast cells were

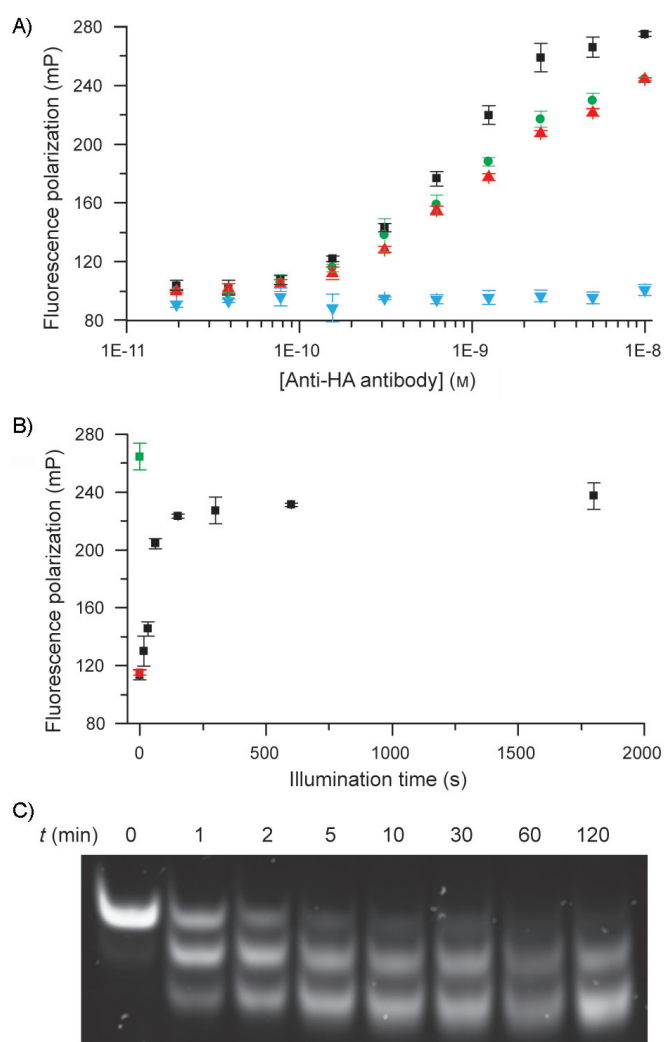


Figure 2. Competitive fluorescence polarization assays showing light-activatable control over antibody activity. A) Titration of anti-HA antibody to 2 nM fluorescently labelled HA peptide in the absence (■) or in the presence of a twofold excess either of monovalent HA peptide (●) or of the bivalent photocleavable lock (▼: before illumination; ▲: after 10 min of 365 nm illumination). B) Restoration of antibody activity as a function of illumination time. Anti-HA antibody (10 nM) was incubated with 2 equivalents of the photocleavable lock and 2 nM of fluorescein-labelled HA peptide. ■: fluorescently labelled HA peptide in the absence of anti-HA antibody, ■: the fluorescently labelled HA peptide with anti-HA antibody in the absence of bivalent peptide-DNA lock, ■: locked antibody. Error bars represent standard deviation, based on three measurements. C) A polyacrylamide gel (15%), stained with SybrGold, showing the decrease in mass upon longer exposure to 365 nm light. A downward shift is observed when either one or both of the peptides is/are cleaved from the dsDNA complex after light exposure.

analyzed by flow cytometry. Figure 3C shows that antibody binding was efficiently blocked by the addition of only slightly more than one equivalent of photocleavable lock. Next, the reversibility of the antibody blockage was assessed by using 1 nM anti-HA antibody pre-incubated with 2 nM of pc-HA₂-DNA. Figure 3D shows that irradiation of the lock with 365 nm light for 30 min resulted in an approximately fivefold increase in antibody labelling of the yeast cells, to a level that is 50% of that observed with the free anti-HA antibody. The incomplete

restoration of antibody binding is due to the presence of 4 nM of monovalent HA peptide cleavage products, which can compete with the HA peptides displayed on the yeast cells for binding to the unlocked anti-HA antibody. A control experiment using anti-HA antibody incubated with 4 nM of monovalent pc-HA peptide indeed shows the same amount of antibody binding to yeast cells as observed after photoactivation of antibody incubated with 2 nM of pc-HA₂-DNA (Figure S4).

A similar, albeit slightly smaller, effect was observed when the lock was incubated with 20 equivalents of a displacer strand for 60 minutes. The displacer strand binds strongly to the 8 bp toehold incorporated in the lock and disrupts the double helix of the lock through toehold-mediated strand displacement. The somewhat lower level of antibody labelling observed after the displacement reaction might be due to a slightly higher affinity of the non-photocleaved monovalent HA peptide, resulting in stronger competition (Figure S3). Indeed, combining light activation and the addition of displacer strand resulted in a level of activation similar to that obtained with photoactivation alone. The pc-HA₂-DNA ligand thus behaves as an OR gate that allows restoration of antibody activity by using either light or a specific oligonucleotide trigger.

In conclusion, a bivalent DNA-peptide ligand has been developed that can be used to cage antibody activity and allows activation of antibody binding under the influence of light. Although proof of principle was provided by using an anti-HA antibody, the molecular design of the lock is generic and applicable to any monoclonal antibody, provided that an epitope or mimotope of sufficient affinity is available. Firstly, these photoactivatable antibodies are attractive research tools, providing the antibody equivalent of the low-molecular-weight ligands developed in photopharmacology.^[23] Possible in vivo applications, such as light-triggered local activation of therapeutic antibodies, can also be envisioned, but would probably require enhancement of the hydrolytic stability of the DNA linker and shifting of the wavelength of the excitation light to the red. Several chemical approaches for increasing the hydrolytic stability of the linker are available, including the use of 2'-MeO- or phosphorothioate-modified DNA, or the use of protein nucleic acids (PNAs) or locked nucleic acids (LNAs).^[24] Similarly, a variety of red-light-activatable photocleavable ligands have recently been reported that could be used to avoid the potential phototoxicity of 365 nm light and to allow deeper tissue penetration.^[25,26]

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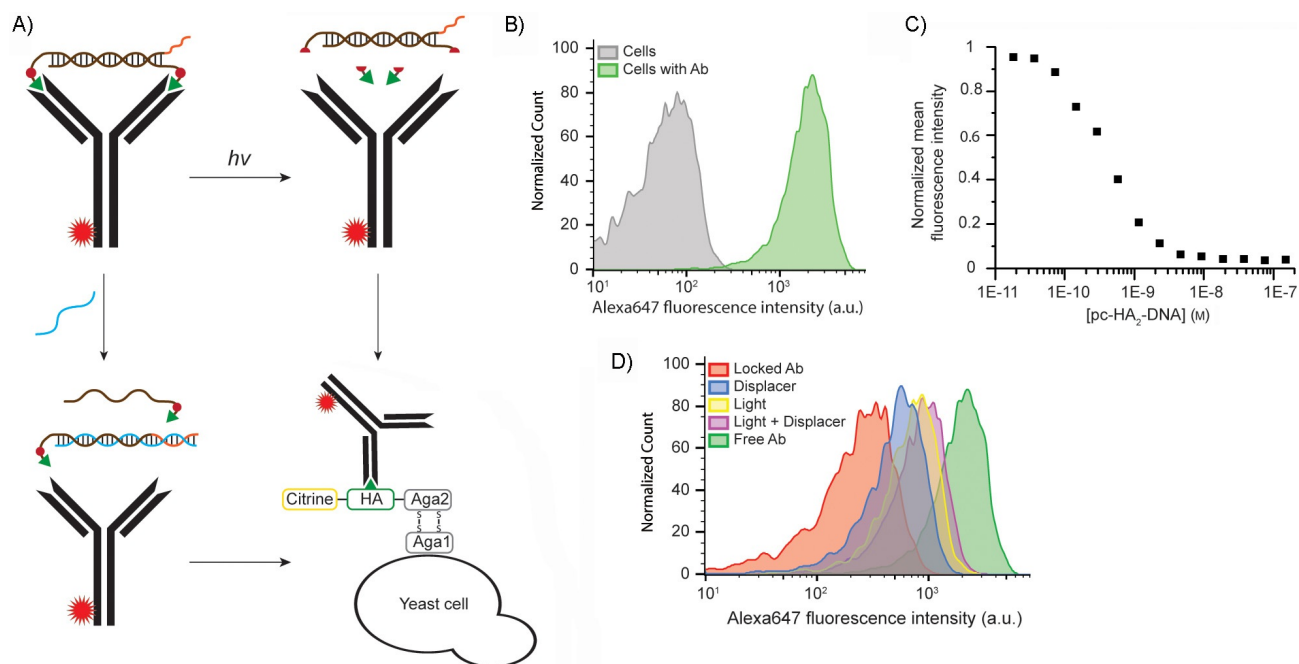


Figure 3. Antibody activation by light and/or toehold-mediated DNA strand displacement recorded by using flow cytometry. A) Yeast surface display. The HA-tag and citrine are displayed as an Aga2 fusion protein on the surface of a yeast cell. The photocleavable lock is used to block an Alexa 647-labelled HA antibody. Upon 365 nm irradiation or addition of a displacer strand, the bivalent lock is converted into two more weakly binding monovalent peptides. The antibody can then bind to the HA-expressing yeast cells, and the fluorescence of the cells can be determined by flow cytometry. B) Histogram of Alexa 647 fluorescence of HA-expressing cells with or without the addition of 1 nM Alexa 647-labelled anti-HA antibody. C) Titration experiment monitoring the binding of anti-HA antibody (1 nM) as a function of the concentration of the photocleavable lock. Fluorescence is normalized to free anti-HA antibody. D) Histogram showing Alexa 647 fluorescence for yeast cells incubated with 1 nM Alexa 647-labelled anti-HA antibody in the presence of 2 nM photocleavable lock. Activation of the antibody is achieved with either 365 nm irradiation, the addition of 20 equiv displacer strand, or both.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: antibodies · bivalent ligands · caged ligands · optogenetics · photolysis

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