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Handcuffed antisense oligonucleotides for light-controlled cell-free expression†

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Developing simple methods to silence antisense oligonucleotides (ASOs) using photocages opens up the possibility of precise regulation of biological systems. Here, we have developed a photocaging strategy based on 'handcuffing' two ASOs to a protein. Silencing was achieved by divalent binding of two terminally photocleavable biotin-modified ASOs to a single streptavidin. These 'handcuffed' oligonucleotides showed a drastic reduction in gene knockdown activity in cell-free protein synthesis and were unlocked through illumination, regaining full activity.

Cell-free protein synthesis (CFPS) systems, which produce functional protein in a minimal environment have found use in synthetic biology to generate DNA logic circuits and synthetic cells for applications in biocomputing and studying living processes. Controlling CFPS with light is very attractive for these applications as it acts orthogonally to most cellular signals, can be applied remotely, and allows for tight spatio-temporal control.[1] A common method of controlling gene expression using light is through macrocyclization of short oligonucleotides (ONs) such as antisense oligonucleotides (ASOs), which silences ASO activity through topological constraint. Through conformational restriction of an otherwise flexible single-stranded ON into an unnatural shape, extended base-pairing into a rigid duplex structure as well as recognition by nucleic acid-binding proteins is hindered. The approach of circularising short ONs has already been successfully used in the silencing of DNA[2–5] and morpholino[6–9] ASOs as well as CRISPR guide RNAs,[10] employing a range of photocages, but has not been applied in CFPS systems. A downside of these approaches is that usually several reaction steps are necessary to yield the desired, circularised ON, and it often takes several steps to synthesise the multifunctional photocage. The circularisation molecules used are also hydrophobic, do not provide additional steric block and do not allow for simple further derivatisation. In addition, the circularised oligonucleotides often need to be purified to remove any unwanted side products after cyclisation.

We wanted to study whether we can use our previously reported photocleavable (PC)-Biotin technology[11,12] together with wild-type streptavidin (wtSA) to form macrocyclized, light-activatable (LA)-ASOs. wtSA provides four binding sites, enabling circular binding of two ONs and thus a high caging density. wtSA is also water soluble and provides steric bulk, due to the size of the protein (53 kDa), allowing for an additional factor in silencing the modified ON. With an eye on future applicability, wtSA allows for further derivatisation to improve cellular uptake, including fusion proteins with HIV-TAT[13] or antibodies[14] and surface modification.[15,16]

To produce circularised, streptavidin-bound ASOs we modified commercially available ASOs that contained 5′-C6-amine and 3′-glyceryl-C3-amine modifications with two different colour (PC)-Biotins (Fig. 1). For modification of the amines, the UV PC-Biotin NHS-ester was commercially available and we have previously synthesised the blue PC-Biotin PFP-ester.[12]

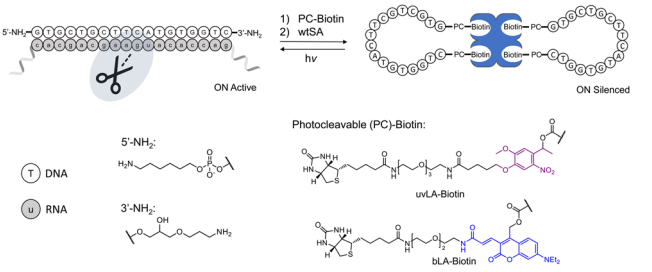

Fig. 1 Synthesis of handcuffed antisense oligonucleotides (ASOs) from dual terminal amine-functionalised precursors using photocleavable (PC)-Biotin and binding to wild-type Streptavidin (wtSA). Circularisation prevents effective hybridisation with its target mRNA, which is recovered upon illumination.

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These doubly biotinylated ASOs were then caged with 0.5 eq. wtSA to form circularised, 'handcuffed' ASOs. We were able to show that these handcuffed ASOs were capable of light-controllable RNase H activity against its target mRNA and light-controllable gene knockdown of CFPS. These handcuffs could also be transfected into living cells and imaged using the intrinsic fluorescence of the photocage.

We initially screened for an RNase H active ASO against *mVenus* (*mV*) that formed a hairpin stem, for ease of macrocyclization to the wtSA. We chose ONs which formed a hairpin with a short stem, as due to the secondary structure and the resulting preorganisation it would have the highest propensity to bind to the same wtSA *via* the adjacent binding pockets. Such a hairpin was also employed previously for the formation of circularised ASOs.[2] To do this we used a python script to check for sequence stretches in the *mV* gene having a length of 16–24 nucleotides (nts) in length and which can form a hairpin stem of 4–6 nts (ESI†). We selected five that fulfilled ASO criteria, purchased the corresponding antisense sequences, and screened those for activity of mRNA degradation with RNase H (Fig. S1, ESI†). We found that ON 252[4] (position 252 in the mRNA of *mV* with a stem of 4 nts) showed the highest activity of the ONs screened. We also modelled the feasibility of the desired macrocyclization, or 'handcuffing', in Chimera, by using published crystal structures of streptavidin with a DNA hairpin appended with our recently reported bLA-Biotin[17] *via* commercial 5′-C6-amine and 3′-C3-glyceryl-amine modifications (Fig. S2, ESI†). This model demonstrated that this 'handcuffing' approach should be feasible, as the distance between adjacent biotin binding pockets is the same as the width of the B-DNA helix (2.0 nm),[18,19] with the added flexibility of the bLA-Biotin linker.

To generate these 'handcuffed' structures, we then used our recently reported blue PC-Biotin PFP-ester[12] and modified the ON 252[4] containing dual terminal amine functionalisations (5′-C6-amine and 3′-C3-glyceryl-amine). The resulting product was purified by high performance liquid chromatography (HPLC, ESI†). Utilising a common method to form macrocycles, we used 3 different dilutions to identify what the effect on the number of higher order structures was, when incubating the ON with 0.5 equivalents of wtSA overnight (Fig. S3, ESI†). Measured using denaturing polyacrylamide gel electrophoresis (PAGE) with staining for the nucleic acid and then the protein, we found that at the lowest dilution condition trialled (0.33 μM ON) the number of higher order structures was lowest, with only minimally more at the intermediate concentration (1.3 μM), yielding what we believed to be the desired, 'handcuffed' oligonucleotides as the major product. Using a protein stain allowed us to confirm that the observed bands were indeed the ON bound to the protein.

Following this initial test, the amino-252[4] ON was also reacted with UV PC-Biotin NHS-ester and purified by HPLC (ESI†) and bound to 0.5 eq. wtSA at high dilution. Both bLA-252[4] and uvLA-252[4] almost exclusively formed a single product, which we expected to be the handcuffed ON (Fig. 2b). It is worth noting that the size difference on the gel of the uvLA-

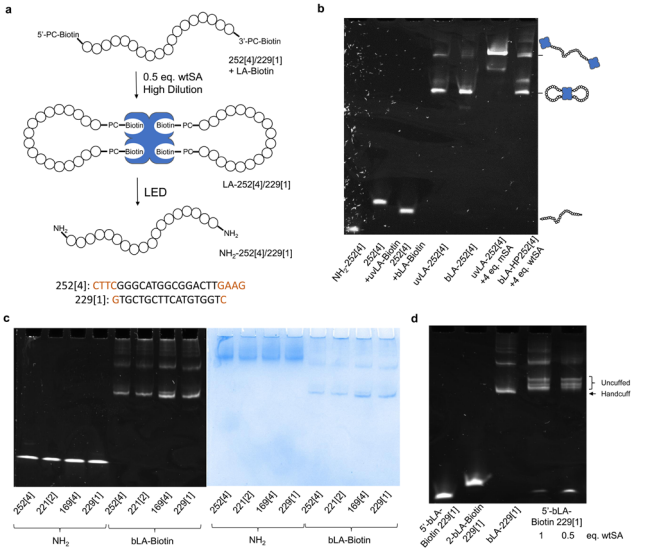

Fig. 2 Formation of handcuff-oligonucleotides (ONs). (a) Formation of handcuffed ONs at high dilution with 0.5 eq. wtSA. The ON can then be released using LED irradiation. (b) Gel-shift assay (8% polyacrylamide) of the PC-Biotin modified and handcuffed 252[4] ONs with wtSA and mSA. (c) Formation of handcuff structures from sequences with lower constraints. (d) Gel shift assay of bLA-229[1] handcuff ON compared to 5′-only modified 229[1] ON with 1 and 0.5 eq. wtSA.

Biotin-modified 252[4] ON and bLA-Biotin-modified 252[4] ON is likely due to the longer linker unit of the uvLA-Biotin, whereas, when bound to wtSA, little/no difference in gel retention is observed between the two different modified ONs, as the size is dominated by the protein. To further investigate the caging and seek additional confirmation of structure, we also incubated the uvLA-Biotin-modified 252[4] with 4 equivalents of mSA (Fig. 2b). As expected, this gave rise to a band with larger gel retention, as this would form a dumbbell-shaped ON with two mSAs, a mSA on each end of the ON. This demonstrated that our 'handcuffed' ON band in the gel only contained a single SA. When adding an excess of wtSA, while the major product was still the same as our 'handcuffed' ONs, the presence of higher order structures, both containing one and two SAs increased (Fig. 2b). Most interestingly, we observed a band in-between the 'handcuffed' ON and the two SA band. This band is likely to be a 1 : 1 ratio of ON to wtSA, as the negative charge of the formed species is approximately half, while only having a small reduction in hydrodynamic radius.

To see if this circularisation approach required the preorganisation provided by the hairpin stem, we used the algorithm to screen for sequences containing two to four base pair hairpins, along with an ASO sequence we have previously optimised that happened to have a single base pair hairpin.[20] Lower constraints on the sequence design would be more desirable, as this would allow for application with previously designed ASOs and we expected the hairpin motif itself might reduce activity. Initially, the ONs with one or two base pair hairpins are unlikely to form hairpins. As expected, the ONs with lower constraints performed better at degrading the target mRNA (Fig. S4, ESI†). We then chose the one (229[1]), two (221[2]) and an additional

four (169[4]) base pair hairpin sequence to test our macrocyclization strategy. All three were modified with the bLA-Biotin, bound to wtSA and analysed by PAGE. Excitingly, all ONs tested formed the handcuffed structure in the same way as bLA-252[4] (Fig. 2c). Therefore, the stem length and hairpin-formation have no influence on the macrocyclization of ONs into handcuffed structures with wtSA. Using a protein stain, we could also confirm the protein-DNA conjugate. To further confirm the desired handcuff structure, we prepared the 5′-only bLA-Biotin modified 229[1] sequence and bound it with 1 and 0.5 eq. of wtSA (Fig. 2d). All bands of this singly biotinylated 229[1], bound to wtSA, had a lower electrophoretic mobility than the major band formed with the doubly-modified ON. This is believed to be due to the larger hydrodynamic radius of the ON-wtSA conjugate if only one terminus binds to the protein. To further confirm the constrained nature of the ON, we incubated the handcuff 229[1] ON with DNase I (Fig. S5, ESI†). The band believed to be the handcuffed ON did not degrade with DNase I, whereas the unmodified ON, as well as the bands corresponding to uncuffed ON, did degrade during incubation, providing further evidence for the macrocylised product.

To investigate the photorelease of the handcuff with light (Fig. 3a), we irradiated the uvLA-252[4] ON with a 365 nm LED at different time intervals (Fig. 3b). Following illumination, bands with a lower electrophoretic mobility than the handcuffed ONs were initially formed before release of the original amino-252[4] ON. The formation of new SA-ON bands with more gel retention provides further evidence that we are forming the most compact handcuffed ON structure, as new bands will be from partial release of an ON from the wtSA, leading to a structure with similar charge and molecular weight but larger hydrodynamic radius. These new bands with lower mobility also resemble those observed with the 5′ only-modified bLA-229[1] ON bound to SA in Fig. 2d. Irradiation of the handcuffed bLA-252[4] ON with 0.5 eq. and 4 eq. of wtSA showed rapid release of the original amino-252[4] ON (Fig. 3c). In both cases the fully handcuffed ON was completely gone following illumination.

When applying both uvLA-252[4] and bLA-252[4] hairpins in an RNase H-mediated mRNA degradation assay (Fig. 4a), both showed little/no mRNA degradation, again demonstrating that these are the handcuffed ON structures (Fig. 4b and c). Upon illumination with UV light (for 10 minutes) or blue light (for 1 minute) the activity of the original ASO was recovered in both cases. When using excess wtSA (Fig. 3c) we still saw effective silencing of the ASO activity, demonstrating that the protein was also providing a steric block to the structures that are not fully handcuffed. Using the dumbbell-shaped ON with mSA, a much smaller difference in activity pre- and post-illumination was observed (Fig. S6, ESI†), with the little difference again demonstrating the steric component of the bound protein. The non-hairpin-forming bLA-229[1] handcuff also showed little/no mRNA degradation prior to irradiation and restoration of activity upon illumination with blue light (Fig. 4d), with an overall higher activity than the hairpin-forming ASOs. This proves that not only is the hairpin not necessary for effective macrocyclization and caging, but is detrimental.

To demonstrate application of these handcuffed structures to CFPS, we tested their efficiency as ‘off’ switches. The bLA-252[4] and bLA-229[1] handcuff ONs were combined with a commercial CFPS system (PURExpress) that was supplemented with RNase H and a DNA template encoding for *mVenus* (Fig. 5a). From initial tests we knew the 229[1] ASO had a higher knockdown activity compared to the 252[4] ASO, therefore, we used a lower concentration (85 nM vs 571 nM) in the CFPS reaction. Even with this difference in concentration, the dual terminal amino-252[4] knocked down expression by 77% (Fig. 5b), whereas the dual terminal amino-229[1] knocked down expression by 90% (Fig. 5c), compared to not adding the ASO. When using the handcuffed ASOs, without illumination, knockdown

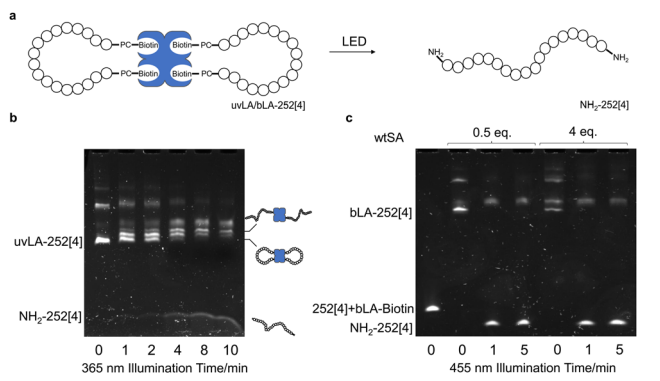

Fig. 3 Uncaging of the handcuff ON. (a) Schematic of the uncaging of the handcuff ON. (b) Photorelease of the uvLA-252[4] handcuffed ON under 365 nm irradiation. (c) Photorelease of the bLA-252[4] handcuffed ON under 455 nm irradiation.

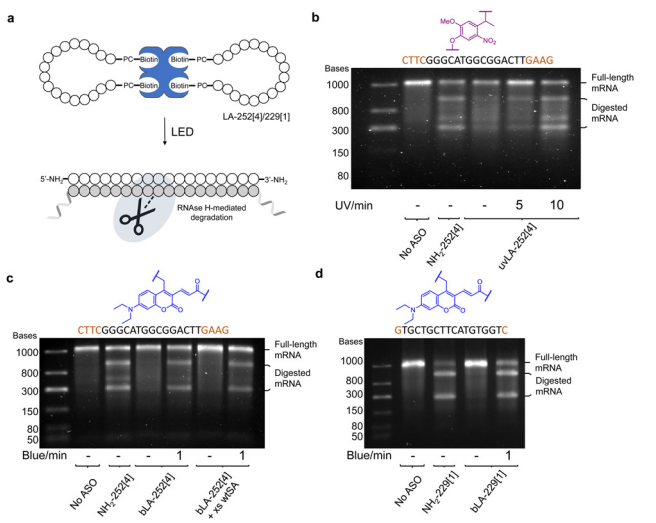

Fig. 4 Control of RNase H activity using the handcuffed oligonucleotides. (a) Photorelease of the ON from the handcuff, eliciting RNase H-mediated degradation of the target mRNA. (b) Light-controlled activity of the uvLA-252[4] handcuff against *mV* mRNA. (c) Light-controlled activity of the bLA-252[4] handcuff with 0.5 and 4 eq. wtSA. (d) Light-controlled activity of the bLA-229[1] handcuff with 0.5 eq. wtSA.

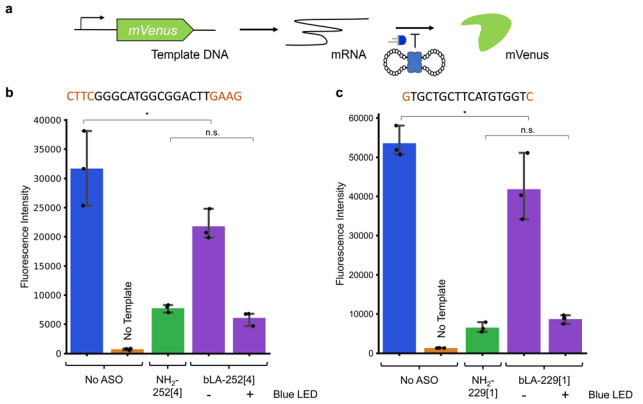

Fig. 5 Control of cell-free protein synthesis (CFPS) using handcuffed ONs. (a) CFPS with the handcuffed ON blocking translation upon illumination. (b and c) Controlled CFPS with blue light using the handcuffed bLA-252[4] ON (b) and bLA-229[1] ON (c). Handcuffing showed a drastic reduction in knockdown activity, which was recovered upon irradiation with blue light. n.s. – not significant (p-value > 0.05). * - significant (p-value < 0.05).

with bLA-252[4] was reduced to 32% (significant, p-value = 0.033) and knockdown with bLA-229[1] was reduced to 22% (significant, p-value = 0.049). The slightly higher residual activity of the 252[4] ON is likely due to its longer size (23 nts), compared to 229[1] (18 nts). In both cases illumination with blue light recovered full activity of the ASO, when compared to the amino-ASO controls (83% for bLA-252[4] (non-significant *vs.* amine-only ON, p-value = 0.051) and 86% for bLA-229[1] (non-significant *vs.* amine-only ON, p-value = 0.954)).

As these handcuffed ASOs may have future application in controlling gene expression in mammalian cells, we tested if the handcuff could be transfected into HEK293T cells. For this, we used the bLA-biotin-modified 229[1] ON by itself as well as the bLA-229[1] handcuff. As an additional advantage of our system, following transfection we could measure the location of our ONs using the intrinsic fluorescence of the coumarin photocage (Fig. S7, ESI†). We found that the DNA/protein conjugate could be effectively transfected. In the future, backbone modifications would be required to show effective gene knockdown in mammalian cells. Together with the nuclease resistance conferred by the circularisation, our ASO handcuffing approach will be applicable for future use in the control of both cell-free and cellular gene expression.

We have developed a simple and effective strategy to form double macrocycles of ONs, handcuffs, using terminal photocleavable biotins and wtSA. These light-controllable handcuffed ONs strongly reduced the RNase H-mediated degradation of targeted mRNA, which could be recovered with illumination of UV or blue light. These were employed to control protein synthesis in a cell-free system using a remote stimulus. Interestingly, it turned out that hairpin-forming ONs are not only unnecessary for effective handcuff-formation and caging, but are in fact detrimental to the activity of the ASO. Our method can be carried out with entirely commercially available reagents (Streptavidin, UV-photocleavable Biotin NHS ester, amino-modified oligonucleotides) and is independent of ON sequence. We also showed that by substituting the photocage, activation can be obtained using alternative wavelengths of light. After attachment of the photocage in a clean and simple procedure, the circularisation is highly effective and does not require further purification. The handcuffed ON also showed stability against a common nuclease and could be transfected into mammalian cells. For future applications, there is a plethora of additional functionality that can be included, through chemical modifications and genetic fusions on the protein for cell-deliverability and improved *in vivo* circulation.

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Conflicts of interest

The authors declare no conflict of interest.

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