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Photocleavable Ligands for Protein Decoration of DNA Nanostructures

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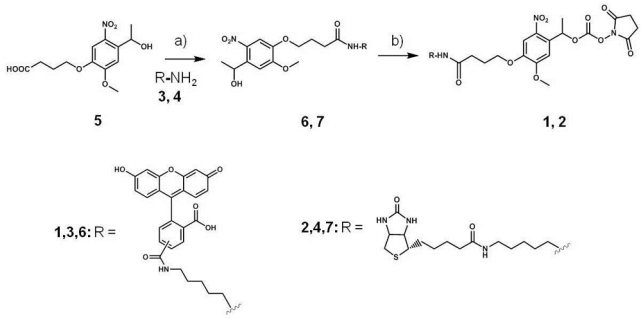
This work describes the synthesis of amino-reactive, photocleavable hapten-modifiers and their application as affinity tags for DNA nanostructures. In particular, N-hydroxysuccinimide-activated linkers containing an α-Methyl-nitroveratryl-butyric acid group and carboxyfluorescein or biotin were synthesized and coupled to alkyl-amino-modified DNA oligonucleotides. The resulting conjugates were then incorporated into DNA origami nanostructures. As demonstrated by electrophoresis and AFM imaging, the functionalized nanostructures were capable to bind cognate proteins which could then be cleaved-off by irradiation. Owing to its modularity, this approach to control protein binding should be useful for a wide variety of functional DNA nanostructures.

The so-called "Scaffolded DNA origami" technique is a method wherein a long single-stranded DNA (ssDNA) scaffold is folded into any desired shape by aid of short synthetic "staple strand" oligonucleotides.[1] This method allows for ready synthesis of finite 2D or 3D DNA nanostructures[2, 3] which can be used as a precisely addressable scaffold offering a single "pixel" resolution of about 6 nanometers.[4] Hence, DNA origami structures are currently being exploited as molecular pegboards for the arrangement of molecular and colloidal components.[4-9] The developments of DNA-protein conjugation techniques are contributing to further advancement of DNA nanostructure technology because proteins possess intrinsic, evolutionary optimized functionality, such as capability for specific molecular recognition and catalytic conversion of ligands and substrates.[10] Significant progress has been achieved recently in the area of protein-decorated DNA nanostructures. For instance, the assembly of synthetic multienzyme cascades on DNA nanostructures yields model systems for the study of spatially-interactive biomolecular networks.[11] Despite these advances, the field of protein-DNA nanostructure bioconjugation is still in its infancy and novel approaches for controllable binding of proteins on DNA scaffolds are urgently needed. The chemical conjugation through cleavable linkers would provide means to control binding and dissociation of target proteins to DNA nanostructures and this strategy has indeed proven successful, e.g., for reductive cleavage of disulfide-containing biotin ligands to selectively control binding of streptavidin (STV) to DNA origami structures.[12, 13] Moreover, photocleavable biotin derivatives are long known for the isolation of biomolecules,[14] and photocleavable phosphoramidite building blocks have been developed to incorporate biotin [15] or fluorescein[16] into short synthetic DNA strands for affinity purification or on-chip DNA sequencing, respectively. We here report the synthesis of photocleavable ligands which posses an active ester moiety to conveniently enable covalent linkage to alkylamino-modified DNA oligonucleotides. The resulting conjugates were incorporated in DNA origami structures to enable ligand-specific binding of cognate proteins to the nanostructure. Photolytic cleavage of the linker led to efficient release of the target proteins.



Scheme 1: Synthetic route to photocleavable ligand modifiers Fsc-PCL (**1**) and Bt-PCL (**2**); a) **3** or **4**, HOBt, EDCxHCl,DIPEA, DMF, rt, 4h or 6h, respectively; b) DSC, DIPEA, DMF, rt, 12h.

As a proof-of-concept for photocleavable ligand modifiers **1** and **2** (Scheme 1), we chose 5,(6)-carboxyfluorescein and D-biotin, respectively, to serve as ligands which are specifically recognized by anti-fluorescein IgG antibodies[17] or STV.[18] 5,(6)-carboxyfluorescein and D-biotin were initially equipped with a six carbon atom spacer by aid of N-boc-1,6-hexanediamine to yield primary amines **3** and **4**, respectively. The amines were then coupled with commercially available α-methyl-nitroveratryl-butyric acid **5**, using standard reagents for peptide condensation. The secondary hydroxyl group in the resulting products **6** or **7** was then activated with a N-hydroxysuccinimide (NHS) group using N,N'-disuccinimidyl carbonate (DSC). Modifiers **1** and **2** were obtained as pure compounds in satisfactory yields of 31% and 62%, respectively. The detailed description of experimental procedures and characterization of compounds is given in the Supporting Information.

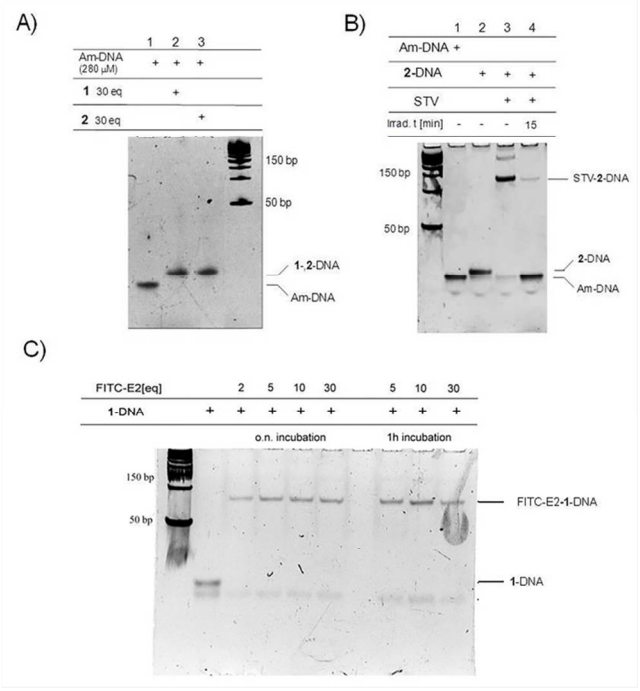


Figure 1: Coupling of ligand modifiers **1** and **2** with an alkylamino-modified DNA (Am-DNA) oligonucleotide (A). This is a 20% urea gel (1x TBE, 220V, 45 min, rt). Note that the shift of the Am-DNA band (lane 1) indicates complete conversion into **1**-DNA and **2**-DNA (lanes 2, 3, respectively). (B): Biotin-containing **2**-DNA binds STV (lane 3) and the linkage is cleaved by irradiation (lane 4). (C): Fluorescein-containing **1**-DNA was allowed to bind anti-fluorescein single-chain antibody FITC-E2. Note that the band of **1**-DNA is retarded due to binding of the antibody.

We then investigated the coupling of ligand modifiers **1** and **2** with an alkylamino-modified 22 nt DNA oligonucleotide, in the following denoted as Am-DNA (Fig. 1). Initial optimization of reaction conditions indicated that 30 molar equivalents of modifiers **1** and **2** were sufficient to quantitatively convert amDNA into the respective conjugates **1**-DNA and **2**-DNA (Figure S1). The conversion is clearly indicated by the shift of the characteristic bands owing to the lowered electrophoretic mobility of **1**-DNA and **2**-DNA (Fig. 1A). Notably, the intrinsic fluorescence of **1** proved to be a useful marker in the analysis of oligonucleotide coupling (Fig. S1B, Fig. S4C). Irradiation of the conjugates with near UV light (350-370 nm) led to efficient cleavage of the ligand from the oligonucleotide (Fig. 1B) and time course experiments revealed that photolytic cleavage was completed in less than 15 min (Figure S2). To investigate whether **1**-DNA and **2**-DNA are functional ligands for cognate proteins, we used the anti-fluorescein single-chain Fv fragment FITC-E2,19 which binds fluorescein with a dissociation constant K_D of about $7.5 \cdot 10^{-10}$ M (Fig.1C), and STV ($K_D \approx 1 \cdot 10^{-15}$ M)[18] (Fig. 1B). Gel shift analyses clearly indicated that the ligand-conjugated oligonucleotides are indeed recognized and bound by FITC-E2 and STV (Fig. 1B, C and S3).

We then investigated whether **1**-DNA and **2**-DNA can be incorporated as functional ligands into DNA nanostructures. To this end, we assembled a rectangular DNA origami structure of approximately 91 x 54 nm^2 from the circular single-stranded 5438 nucleotide plasmid ss109Z5[20] and a set of 180 staple strands. The latter contained six staples which were conjugated with **1** prior to assembly of the origami structure (Fig. S4) The nanostructure was designed to contain the **1**-modified staples at edge positions (green dots in Fig. 2A) to simplify AFM analysis. For control purposes, a similar origami structure (denoted as Fsc-origami) was assembled which contained the six staples conjugated with fluorescein through a non-cleavable linker. Electrophoretic analysis revealed a single band for both origami structures, indicating that essentially a single origami species resulted from the assembly reactions (Fig. 2B). These results were confirmed by AFM analysis which revealed almost exclusively intact rectangular DNA nanostructures (Fig. 2C).

The two origami structures were tested for their capability to bind whole IgG anti-fluorescein antibodies. To this end 35 molar equivalents of anti-fluorescein IgG were added to a solution containing either **1**-origami or Fsc-origami. Both mixtures were aliquoted and one of each aliquot was irradiated with near-UV light for 20 min. The four samples were then analyzed by AFM. Representative AFM images are shown in Fig. 2C. The images clearly show the integrity of the rectangular nanostructures with IgG molecules tethered to one edge of the structures. On average, about three IgG molecules were bound to the origami, presumably due to bivalent binding of IgG to two adjacent fluorescein ligands. While no significant differences could be observed for samples prior to irradiation (left panel of Fig. 2C), treatment with near-UV light led to complete disappearence of IgG in the case of **1**-origami whereas Fsc-origami-IgG constructs remained unaffected (right panel of Fig. 2C). These results clearly demonstrate the functionality of **1** and **2** as ligands for controllable binding of proteins onto DNA nanostructures.

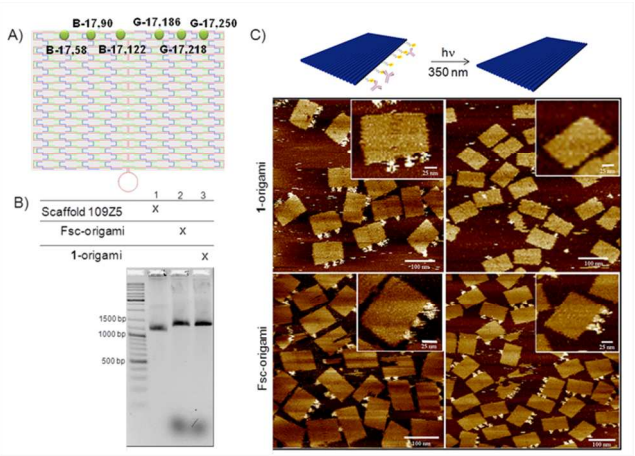


Figure 2: DNA origami structures bearing photocleavable ligand **1** or non-cleavable fluorescein ligands. A) Schematic illustration of origami design. The green dots indicate the positions where six staples modified with either **1** or non-cleavable fluorescein ligands were incorporated. B): Electrophoretic analysis of DNA origami structures containing either **1**- (lane 3) or non-cleavable fluorescein origami (lane 2). Note that both structures reveal a single band with a lower electrophoretic mobility than the single-stranded scaffold strand (lane 1). Running conditions: 1,5% agarose gel, 1X TBEMg, 80 V, 1,5 hrs, 4°C, EtBr staining. (C): AFM images obtained from anti-fluorescein IgG-

conjugated **1**-origami (top) and Fsc-origami (bottom) prior (left) or subsequent (right) to irradiation with near UV light. Note the disappearence of proteins in the upper right image.

Conclusions

In conclusion, we here demonstrated that small-molecule ligand appended to DNA nanostructures through a photocleavable linker provide an efficient means to control the binding of cognate proteins to the nucleic acid scaffold. Owing to the facts that more complex scaffolds are readily available by design of 2D and 3D DNA origami structures and that the modular scheme for synthesis of photocleavable ligands described in this work can easily be adopted to other ligands bearing a primary amino group, we anticipate that further exploitation of this approach might lead to more complex, functional supramolecular nucleic acid architectures. It should also be noted that AFM-based analysis is well established for quantitative assessment of binding and release of proteins because it allows for statistical analysis along with single-molecule sensitivity.[12, 13] Therefore, the here described approach could be extended to photocleavable affinity tags for controlling the binding of enzymes to arbitrary positions on origami scaffolds to enable quantitative studies of spatially-interactive biomolecular networks.[11]

Notes and references

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† Electronic Supplementary Information (ESI) available: Synthesis and characterization of small-molecule compounds, general procedures for DNA conjugation linkers, procedures and sequence information regarding DNA origami assembly and characterization. See DOI: 10.1039/b000000x/

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