Solid-Phase Synthesis and Purification of Protein–DNA Origami Nanostructures

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Abstract: We present a facile method for the combined synthesis and purification of protein-decorated DNA origami nanostructures (DONs). DONs bearing reductively cleavable biotin groups in addition to ligands for ligation of recombinant proteins are bound to magnetic beads. Protein immobilization is conducted with a large protein excess to achieve high ligation yields. Subsequent to cleavage from the solid support, pure sample solutions are obtained which are suitable for direct AFM analysis of occupation patterns. We demonstrate the method's utility using three different orthogonal ligation methods, the "halo-based oligonucleotide binder" (HOB), a variant of Halo-tag, the "SpyTag/SpyCatcher" (ST/SC) system, and the enzymatic "ybbR tag" coupling. We find surprisingly low efficiency for ST/SC ligation, presumably due to electrostatic repulsion and steric hindrance, whereas the ybbR method, despite its ternary nature, shows good ligation yields. Our method is particularly useful for the development of novel ligation methods and the synthesis of mechanically fragile DONs that present protein patterns for surface-based cell assays.

Since the invention of the "scaffolded DNA origami" technique,[1] DNA origami nanostructures (DONs) are becoming increasingly popular for a variety of applications.[2] Their use as "molecular pegboards" for the arrangement of non-nucleic acid components is of particular interest because typical DONs possess an addressable surface area of a few thousand nm^2 with a single "pixel" resolution of about six nanometers.[3] Since proteins have intrinsic, evolutionary optimized functionalities, such as capability for specific binding and catalytic conversion of ligands and substrates, the decoration of DONs with proteins opens up the door to applications in biosensing and nanofabrication of supramolecular constructs.[4] For example, antibody-modified DONs are promising reagents for applications in nanomedicine[5] and synthetic multienzyme cascades arranged on DNA nanostructures are currently being explored as model systems for spatially interactive biomolecular networks and novel biocatalytic systems.[6]

Proteins are difficult to immobilize because their tertiary structures are often sensitive to chemical manipulations which can lead to denaturation and loss of activity. Therefore, chemically mild, orthogonal coupling procedures are required to enable the efficient site-selective coupling of various different proteins with DONs.[4] To this end, methods based on recombinant protein tags,[7] Zn-finger proteins[8] or supramolecular interactions[9] were developed that allowed for the direct coupling of proteins on the DON surface. Alternatively, DONs bearing single-stranded capture strands can be hybridized with oligonucleotide-protein conjugates that are accessible by a variety of methods.[4,10]

Both strategies require the use of a stoichiometric excess of the protein-of-interest (POI) in order to achieve the highest possible coupling rates. This approach usually necessitates the separation of excess unbound proteins to avoid artifacts when quantifying the specific activity of the supramolecular construct. Likewise, the development of new coupling methods requires efficient purification of assembled DON-protein constructs to quantify coupling efficiencies by AFM analysis. Previously used methods based on electrophoresis, ultrafiltration and chromatography[11] or Ni-NTA-affinity tag purification[12] are often not applicable when it comes to purification of fragile DONs decorated with sensitive proteins.[7b] Högberg and coworkers have recently evaluated a panel of purification methods for protein-functionalized 18-helix bundle DONs.[11c] However, these 3D structures are more stable than mechanically sensitive 2D structures and the binding of the proteins was only achieved by hybridization of previously synthesized oligonucleotide–protein conjugates. Since direct ligation of proteins on the surface of DONs brings advantages in terms of synthesis effort and costs, and since flexible 2D DONs are important reagents for surface-based cell assays,[13] there is still a great need for a convenient and robust method to prepare and purify fragile DONs on which proteins have been immobilized by chemoselective coupling methods.

We here describe a readily applicable method for the purification of protein-decorated DONs. Our method is based on robust (strept)avidin–biotin interaction and enables rapid assembly and purification of protein-decorated origami structures produced by orthogonal coupling methods. As shown in Figure 1, DONs bearing orthogonal coupling groups, such as small-molecule tags for self-ligating proteins, are equipped at their edge with biotin groups connected to the origami using cleavable linkers. (Strept)avidin-coated magnetic microbeads

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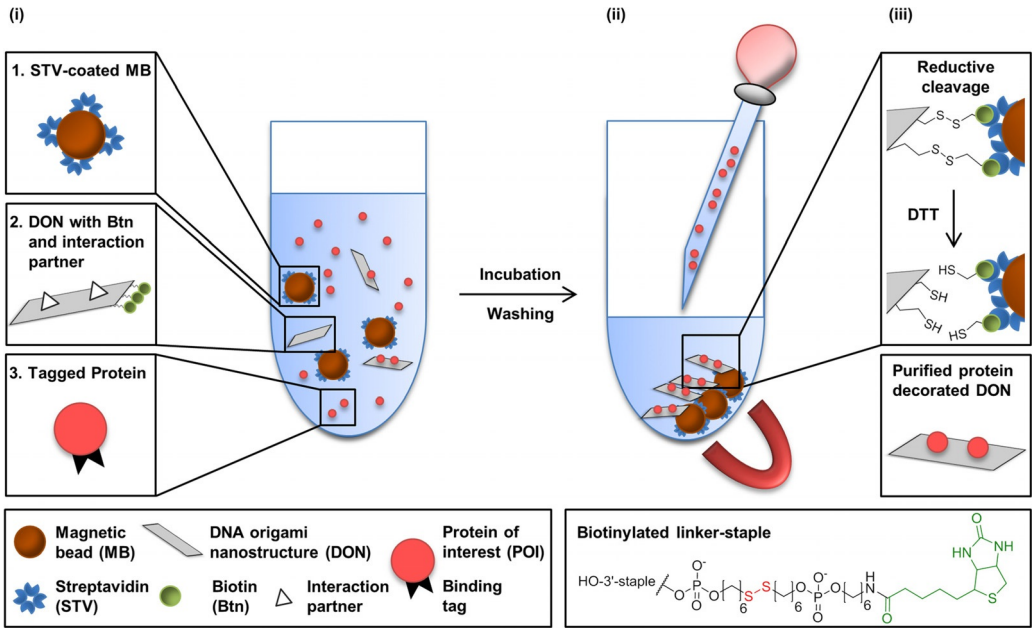

Figure 1. Schematic illustration of the magnetic microbead (MB) based purification of protein decorated DNA origami nanostructures (DONs). (i) In a one pot procedure, streptavidin (STV) coated MBs are mixed with a protein of interest (POI) and DONs bearing reductively cleavable biotin (Btn) staples at their edge and small molecule tags on their surface for orthogonal POI ligation, respectively. (ii) MBs with bound DONs are purified by magnetic separation. (iii) Isolation of decorated DONs is achieved by reductive cleavage with dithiothreitol (DTT). The inset at the bottom shows the structure of the cleavable biotin linker staple.

(MBs) are mixed with the DONs and a large excess of recombinant fusion proteins that covalently bind with the small-molecule tags. Magnetic separation of the beads and washing is used for complete removal of unbound and loosely attached proteins. Treatment of the beads with a reductant then leads to cleavage and enables the separation of highly concentrated purified protein-decorated DONs. We demonstrate the utility of the method by the synthesis of DONs modified through three different orthogonal coupling systems.

To establish the system, as a positive control, we firstly used the "halo-based oligonucleotide binder" (HOB), a self-labeling protein tag (293 amino acids) that forms a covalent bond with small-molecule chlorohexane (CH)-ligands in a similar fashion as the regular Halo-tag protein, which is commonly used for imaging in cell biology.[14] HOB was genetically engineered to bind to CH-ligands attached to DNA oligonucleotides and DNA nanostructures with a significantly higher efficiency than Halo.[15] Then we used two coupling systems, which had not yet been tested for the direct modification of DONs with proteins. On the one-hand, we tried to immobilize recombinant proteins by using the "SpyTag/SpyCatcher" system that consists of the 113 amino acid SpyCatcher (SC) protein, which generates a covalent isopeptide bond between one of its lysine residues and an asparagine residue of the 13 amino acid SpyTag (ST) peptide (Ala-His-Ile-Val-Met-Val-Asp-Ala-Tyr-Lys-Pro-Thr-Lys).[16] On the other hand, we investigated the performance of the "ybbR tag" coupling system, which is based on the Sfp (surfactin production) phosphopantetheinyl transferase-catalyzed ligation of proteins bearing the 11-residue ybbR peptide (Asp-Ser-Leu-Glu-Phe-Ile-Ala-Ser-Lys-Leu-Ala) with small-molecule-Coenzyme A (CoA) conjugates.[17] While this system has previously been used for the covalent attachment of CoA-modified oligonucleotides to ybbR-tagged proteins,[18] the direct ligation on the surface of DONs has not yet been explored.

To test the suitability of our method for protein decorated DONs, we assembled a $54 \times 92\ nm^2$ rectangular plate DNA origami from the single-stranded 5438 nt template 109Z5.[19] All DONs contained five Cy5-labeled staple strands to enable fluorescence detection. Further, the DONs contained three distal biotin groups attached through cleavable linkers at the edge and three or four small-molecule ligands for ligation with appropriate fusion proteins. The chemical modification of staple strands with the different ligation tags for POI immobilization was achieved by standard methods using activated ligand precursors and heterobispecific crosslinkers (Figure S1). The modified staples were analyzed by gel electrophoresis and their functionality for coupling with the complementary tagged proteins was confirmed by gel-shift analyses (Figure S2, S3). Furthermore, staples modified with the cleavable biotin linker were tested for their reductive cleavage with dithiothreitol (DTT) and their binding capacity for streptavidin (STV) (Figures S4, S5). Likewise, we confirmed by AFM and electrophoretic analyses that the assembled DONs bearing the three cleavable biotin linkers can bind and release STV (Figures S6, S7).

For an initial assessment of the MB-based purification and as a positive control, we tested the HOB coupling system, which has previously been demonstrated to deliver high ligation yields of about 75% for enzymes of comparable and even larger size as the enzyme Gre2.[15] We used DON-1 that contained three CH-ligands to enable the direct ligation with the enzyme Gre2 that was genetically fused with the HOB domain

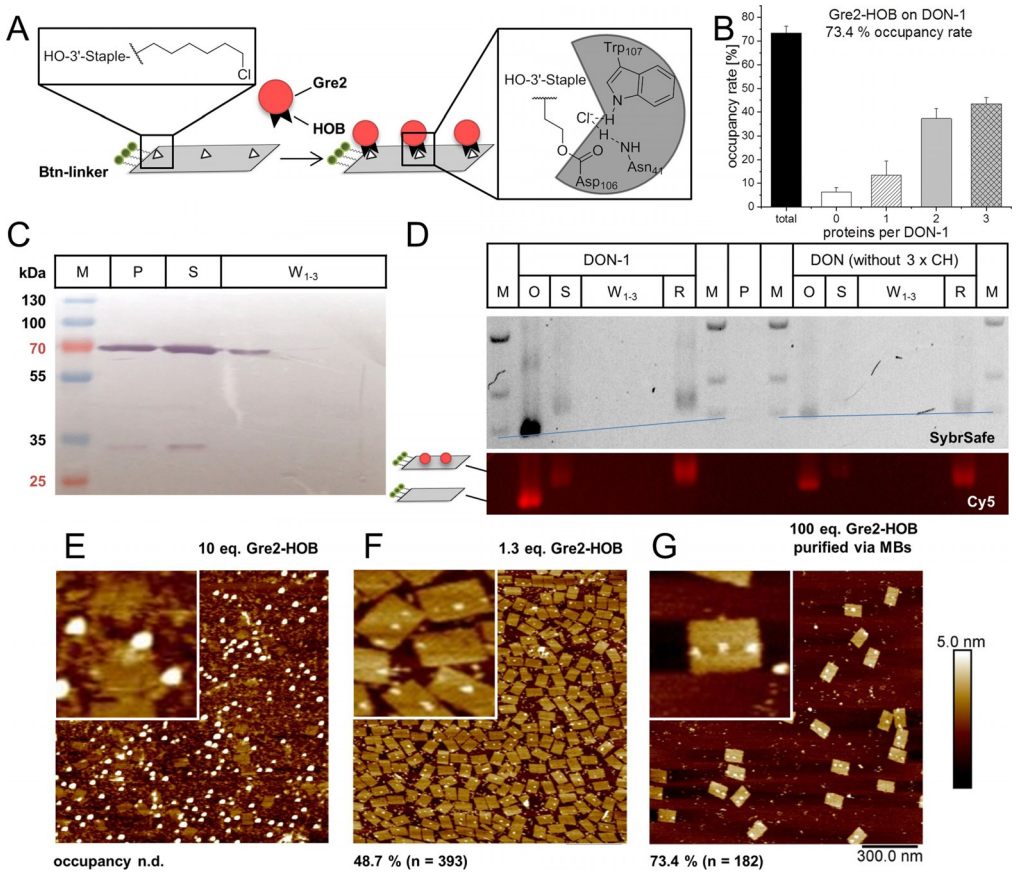

Figure 2. Immobilization of Halo based oligonucleotide binder (HOB) fusion protein Gre2 HOB. A) Schematic illustration of the HOB immobilization and the coupling mechanism of the HOB tag with the DON appended chlorohexane (CH) ligand. B) Statistical analysis of protein occupancy rates of MB purified DON 1. C) Western blot analysis of Gre2 HOB present in the supernatant (S) and wash fractions (W1 3) of the MBs, as compared to the original amount of the protein (P) prior to bead extraction. Note that the absence of bands in the last wash fraction W3 indicates the complete removal of all unbound proteins after the washing. This is a PVDF membrane obtained by blotting of a 12% SDS PAGE (1st antibody (AB): mouse anti His tag, 2nd AB: goat anti mouse AP (al kaline phosphatase)). Marker (M): PageRuler™ plus protein ladder. D) Electrophoretic analysis of the bead based assembly/purification method. Almost the entire original amount of DON (O) is bound to the beads, as indicated by the corresponding weak band obtained from the supernatant (S). Lanes denoted W1 3 show the wash fractions and lane R illustrates DON 1 recovered after reductive cleavage. Note the lower electrophoretic mobility of the protein loaded DONs (lanes S, R) as compared to untreated DONs (O). The samples on the right hand side were obtained from a control with DON lacking the three CH li gands. The slight shift in S presumably stems from unspecific binding. Note that the running front of the gel is slightly curved, as indicated by the blue marker lines. The changes in DON electrophoretic mobility are visible also from fluorescence imaging of the Cy5 labeled DONs (lower panel, the complete gel is given in the supporting information Figure S8C). This is a 1% agarose gel, run time 4 h, 80 V, 4 °C, analyzed by SybrSafe staining (top) and Cy5 fluorescence (bottom). Marker (M): extended bp ladder (New England Biolabs). E) AFM analysis of DON 1 mixed with 10 equiv. Gre2 HOB per DON 1 binding site. Note that no AFM analysis is possible due to high protein background. F) Excess of only 1.3 equiv. of Gre2 HOB allows AFM analysis but leads to a low occupancy rate, whereas on bead coupling with a large excess of protein and subsequent cleavage leads to pure protein DON samples with a high occupancy rate (G).

(Figure 2A). In a typical reaction, 50 μg STV-coated MBs were mixed with 0.5 pmol DON-1 and 150 pmol (corresponding to 100 molar equivalents per DON binding site) of the Gre2-HOB. Subsequent to incubation for 2 h, the MBs were collected with a magnet and thoroughly washed to remove all unbound protein (Figure 2C). Reductive cleavage of the biotin linker was achieved by addition of elution buffer that had previously been optimized in preliminary tests to contain 100 mM DTT (Figure S8). Electrophoretic analysis confirmed that reductive cleavage of DON led to efficient recovery of the Gre2-HOB-modified DON (Figure 2D) with isolated yields of approximately 65%. Importantly, the obtained protein-DON samples were of very high purity that enabled the direct analysis by AFM. This is remarkable because even small amounts of unbound protein usually make AFM analyses very difficult if not impossible (Figure 2E, Figure S12). In agreement with an earlier study,[15] a small excess of 1.3 equivalents of protein allows for AFM analysis of crude coupling products but only affords low occupancy rates (about 49%) (Figure 2F). In contrast, on-bead coupling with a large excess of protein followed by subsequent washing and cleavage yields pure protein-DON samples with high occupancies of about 73% (Figure 2G).

We then used the bead-based method for the assessment of novel methods for directional protein ligation onto DON surfaces. In a first example, we investigated the utility of the "SpyTag/SpyCatcher" (ST/SC) system. To this end, alkylamino-modified staples were covalently modified with the ST peptide (Figures S1–S3) and then used for assembly of DON-2 that contained four ST-ligands on its surface (Figure 3A). As a test protein, we used a variant of the enhanced green fluorescent pro-

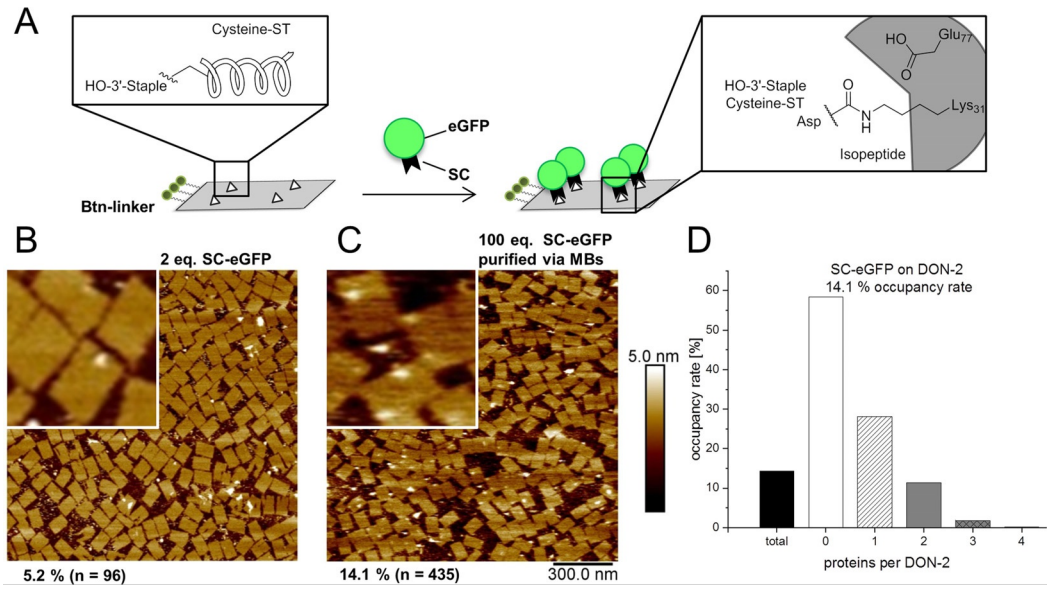

Figure 3. Ligation of SpyCatcher (SC) fusion protein onto DON 2 surface. A) Schematic illustration of the immobilization of SC eGFP fusion proteins on SpyTag (ST) modified DON 2. Covalent coupling is achieved by formation of a covalent isopeptide bond between the ST and SC domain. B) In the case of 2 equiv. SC eGFP per binding site, direct AFM analysis could be achieved to reveal an occupancy rate of 5.2%. C) Incubation with 100 fold excess of protein led to an increased occupancy rate of 14% after purification. D) Bar diagram of the statistical analysis of AFM images indicating the numbers of proteins bound per DON 2.

tein (eGFP) genetically fused with the SC domain.[20] Initial analysis of the coupling reaction of unpurified samples by gel electrophoresis suggested that the ligation takes place only to a very small extent (Figure S9). However, the bead-based coupling method enabled the detailed AFM analysis of this poorly running ligation and clearly showed that occupancy densities of approximately 14% were achieved (Figure 3B–D). The low efficiency of the "on-DON" ST/SC ligation was surprising because protein–protein coupling is near quantitative[16] and also test reactions with oligonucleotides worked well (coupling rates of approx. 68%, Figure S3B). Hence, we hypothesize that "on-DON" conjugation is impaired by steric hindrance in combination with electrostatic repulsion between the large surface of the SpyCatcher-eGFP (SC-eGFP) fusion protein and the negatively charged origami surface. Based on the crystal structure analysis of the ST/SC complex,[21] consideration of the surface charges indeed suggests that for a productive approach of SC-eGFP and ST, negatively charged parts of the protein must be brought into close proximity to the negatively charged DNA surface (Figure S10).

As a further example of the usefulness of our method for the evaluation of approaches for protein ligation on DONs, we investigated the "ybbR tag" coupling system (Figure 4). To this end, staples were functionalized with CoA (Figure S1, S2) and then used to assemble DON-3 which contains four CoA-ligands on its surface (Figure 4A). As a model protein, we used eGFP that was genetically modified with the ybbR peptide tag at its C-terminus. Initial electrophoretic analysis of the coupling reaction of unpurified samples clearly indicated successful protein ligation on the DON surface (Figure S11). No direct assessment of coupling yields by AFM was possible from the crude reaction mixtures due to the high background of non-specifically adsorbed proteins on mica, even when only two molar equivalents of proteins were used (Figure 4B, see also Figure S12). In contrast, the bead-based coupling/purification method yielded a pure sample solution that could be investigated directly with AFM (Figure 4C). Statistical analysis of the AFM images revealed occupancy densities of approximately 56% (Figure 4D). This ligation efficiency is remarkable because the coupling on the DON surface must take place in a ternary heterogeneous reaction between the DON surface-tethered CoA and the two bulky proteins, ybbR-eGFP and Sfp.

In summary, we have developed a convenient and robust method for the combined synthesis and purification of protein-decorated DONs. The method provides high coupling yields, even in cases where the ligation of proteins on the DON surface only occurs with moderate conversion. While we observed unchanged enzymatic activity in the case of Gre2 (Figure S13) attention must be paid to the point that the reductive cleavage step may lead to damage to sensitive proteins. On a case-by-case basis, this could be avoided by using milder reducing agents (e.g. TCEP (tris(2-carboxyethyl)phosphin), Figure S8). Of particular importance is that the method provides sufficient pure samples to allow direct analysis of occupation patterns using AFM. Since the binding of the DONs to the beads takes place through the robust STV-biotin interaction, the method is efficient, so that isolated yields of the protein-decorated DONs were typically in the range of 65% of the amount of origami used. However, our method is not suitable for producing STV-bridged protein patterns on DONs. This limitation could be overcome by alternative bead-binding/release systems, for example, based on DNA hybridization and strand displacement mechanisms.[11c] Even in the current version, the method is of great value for the further development of the

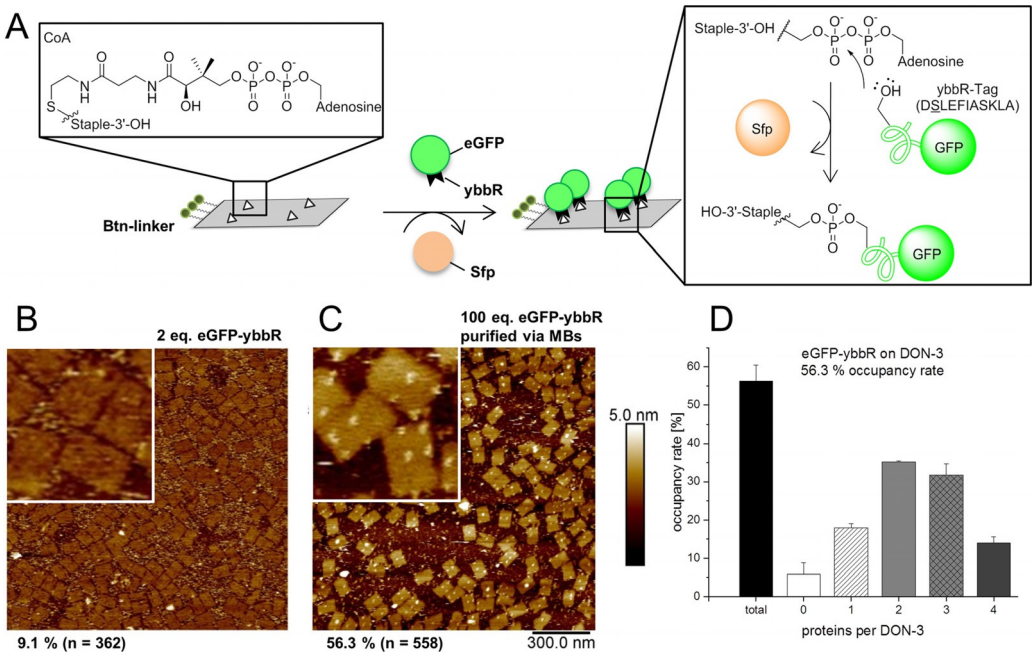

Figure 4. Enzymatic ligation of ybbR tagged eGFP onto DON 3 surface. A) Schematic illustration of the phosphopantetheinyl transferase (Sfp) mediated liga tion on the surface of DON 3 bearing four coenzyme A (CoA) modified staple strands. As detailed in the inset, Sfp catalyzes the nucleophilic attack of the serine residue of the ybbR peptide at the phosphodiester bond of CoA modified oligonucleotides. B) In the case of 2 equiv. eGFP ybbR per binding site, direct AFM analysis could be achieved to reveal an occupancy rate of 9%. C) Bead based coupling/ purification with a 100 fold excess of eGFP ybbR led to an increased occupancy rate of 56%. D) Bar diagram of the statistical analysis of AFM images indicating the numbers of proteins bound per DON 3.

field, as it enables new ligation methods to be evaluated on mechanically flexible and fragile origami structures, which have proven their utility for surface-based cell assays[13] and fundamental research in biocatalysis.[4,6d,22]

Experimental Section

Experimental details can be found in the Supporting Information.

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Conflict of interest

The authors declare no conflict of interest.

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