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Clickable Tyrosine Binding Bifunctional Linkers for Preparation of DNA−Protein Conjugates

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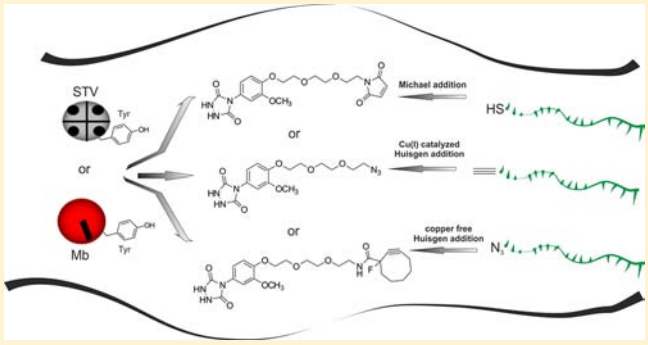
Supporting Information

ABSTRACT: We have prepared bifunctional linkers containing clickable functional groups that enable preparation of protein−DNA conjugates through binding onto tyrosine residues. Mild conjugation strategy was demonstrated using two proteins, streptavidin(STV) and myoglobin (Mb) and it resulted in conjugates with preserved functionality of both the proteins and DNA strands. Furthermore, we show that protein−DNA conjugates can be successfully immobilized onto solid surface containing complementary DNA strands and the enzymatic activity of Mb−DNA conjugates is even higher than that of corresponding conjugates prepared through Lys binding.



INTRODUCTION

The past decade has brought rapid developments in the use of DNA as a structural element to build novel architectures[1−3] and aide the bottom-up fabrication of nanostructure-based systems.[4] Due to its physio-chemical stability, accessibility through chemical synthesis, and high fidelity molecular recognition between complementary strands, DNA oligomers can efficiently be used as structure directing agents for assembly and immobilization of different nanosized elements and have found numerous applications in bioanalytics[5] and biosensing,[6−8] new material design[9] and surface modification.[10] As a mild and reversible method of immobilization, methodology based on DNA hybridization (DNA directed immobilization)[11] is particularly suitable for precise positioning of the proteins on the nanometer scale and design of devices exploiting the versatile protein functions.[12,13] However, one of the main requirements for DNA directed assembly of proteins is availability of functional DNA−protein conjugates.[14] Different approaches, both noncovalent and covalent, are available for their preparation and the choice of the method largely depends on the type of the protein of interest. It is important to keep in mind that it is still challenging to choose a method that would enable the control over both the stoichiometry and regioselectivity of DNA coupling.

Most common noncovalent coupling methodologies are based on streptavidin (STV)−biotin interaction[15] or use of protein tags such as His tag.[16] Another approach applicable to a class of cofactor containing proteins involves the replacement of noncovalently bound natural cofactor with DNA modified, during the process known as cofactor reconstitution, which is applicable to heme[17,18] and flavin[19] containing proteins. However, noncovalent approaches often result in the protein−DNA complexes sensitive to the environmental conditions and prone to dissociation, which renders them unsuitable for studies under cellular matrix conditions.

More robust covalent linkage provides chemical stability and until now, the most widely used methodology for preparation of DNA−protein conjugates has been based on the use of bifunctional linkers to enable cross-linking of thiol or amine modified DNA single strands with appropriate amino acid residues on the protein surface.[20] Such covalent methods help to circumvent the inherent instability problems of the noncovalent complexes, although they often do not result in stoichiometrically well-defined conjugates when native, non-genetically modified proteins are used, which often contain a larger number of amino acid side chains available for modification. Other approaches such as expressed protein ligation,[21] Huisgen 1,3-dipolar cycloaddition,[22] Staudinger ligation,[23] keton−aminooxy[24] or enzymatically aided reactions[25,26] require either addition of bioorthogonoal functional groups through Lys or Cys or introduction of artificial amino acids.[27] For certain classes of proteins the introduction of common affinity protein tags or artificial amino acids is a complex process, which often results in low yields of desired protein and the loss of activity. On the other side, modification through Cys might not be feasible due to the important structural role of Cys-Cys disulfide bridges, and the modification of Lys might affect the distribution of the surface charges and influence the inherent protein−protein interactions. Furthermore, there are often many Lys groups present

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in proteins, which make the control over stoichiometry and stereospecificity a real challenge.

Therefore, we were interested in extending DNA conjugation methodology to other amino acid residues in order to increase the applicability of the DNA directed assembly to protein classes where other modification methods are not suitable. Herewith, we present the use of the clickable bifunctional linkers to prepare DNA−protein conjugates through tyrosine (Tyr) residue modification using a model proteins streptavidin (STV) and myoglobin (Mb). Although strategies exist to modify these proteins through Lys residues, Mb is extensively used in nanotechnology and we are interested in enabling the multimodal modifications of the protein shell in design of artificial enzymes in future applications.

EXPERIMENTAL PROCEDURES

Materials and Instrumentation. All chemicals were purchased from Sigma-Aldrich and used without further purification. Fast protein liquid chromatography (FPLC) was performed using Äkta explorer system with Mono Q 5/50 anion exchange column (GE Healthcare, Germany). Gel filtration NAP5 and NAP 10 columns and Vivaspin filtration columns were also purchased from GE Healthcare (Germany). UV−vis spectra were obtained using VARY 300 Scan UV−visible Spectrometer (Varian Inc.). Thiol modified ssDNA (**shD**) was obtained from Sigma Aldrich (Germany) and azide and alkyne modified ssDNA (**azD** and **alkD**, respectively) from Atdbio (UK). DNA sequences are given in the Supporting Information. The microtiterplates (MTPs) for kinetic measurements in solution were purchased from NUNC (Denmark), while the DDI was realized in NUNC high absorption MTPs from Thermo Fischer Scientific (Germany).

Synthesis of Bifunctional Linkers BL-0, BL-1, BL-2, and BL-3. Due to the extensive synthetic procedure, the details on the synthesis and characterization of biofunctional linkers is given in Supporting Information (SI).

Coupling of BL-1, BL-2, BL-3, to L-Tyrosine. L-Tyr solution in ddH_2O (ca. 2.67 mM, 1.38 μmol L-Tyr used) was added to freshly activated linker solution (2.84 μmol linker treated with 2.84 μmol pyridine (Pyr) and 2.84 μmol of *N*-bromosuccinimide (NBS) in DMF for 5 min on ice) and incubated for 1 h at RT. After the completion of reaction (followed by TLC), the solvent was removed under vacuum and the products were characterized with Fast Atomic Bombardment (FAB) MS. HRMS (FAB): BL-1_Tyr $C_{28}H_{32}N_5O_{11}$ $[M + H]^+$: theor.: 614.2096; found: 614.2098; BL-2_Tyr $C_{24}H_{30}N_7O_9$ $[M + H]^+$: calc: 560.2103; found: 560.2105; BL-3_Tyr $C_{33}H_{41}FN_5O_{10}$ $[M + H]^+$: calc: 686.2; found: 686.2.

Activation of BL-1, BL-2, and BL-3 and Protein Coupling. 2.5 μmol of **BL-1**, **BL-2**, or **BL-3** [62.5 mM solution in DMF] were activated with 2.5 μmol Pyr and 2.5 μmol NBS in DMF for 5 min on ice. A rapid color change from colorless to red could be observed (SI, Figure S3B). Freshly activated bifunctional linkers were used for the protein coupling: 0.12 μmol of STV [300 μM] or 0.12 μmol Mb [300 μM] in 1× PBS buffer (pH = 7.1) were incubated with a 10-fold excess [1.2 μmol] of corresponding activated bifunctional linkers for 15 min at RT (ratio of DMF: ca. 10%). After incubation, the solutions were treated with size exclusion NAP 5 and NAP 10 columns to remove the small and nonreacted molecules. The modified proteins were used for ssDNA and fluorophore coupling immediately.

Fluorophore Coupling to STV and Mb. 0.8 μmol of activated **BL-1**, **BL-2** and **BL-3** [80 mM in DMF] were added to 20 nmol of STV or Mb in 1× PBS buffer (pH = 7.4), respectively, and incubated for 15 min at RT (DMF ratio: ca. 5%). The reaction solutions were treated with gel filtration NAP 5 columns to remove nonreacted small molecules. In the next step, 0.4 μmol of the thiol fluorophore acetylmercaptosuccinoyl-aminofluorescein (SAMSA), azide modified fluorophore (AzF), or cyclooctyn-fluoresceine (COF) was added to freshly prepared corresponding bifunctional linker−protein conjugate solutions, incubated overnight at 4 °C in the dark, excess fluorophores removed by gel filtration and concentrated using Vivaspins filtration systems (cutoff: 5 kDa (Mb), 10 kDa (STV)) to a final volume of ca. 200 μL. Successful coupling of the fluorophores (SI, Scheme S7) onto the protein was confirmed by gel electrophoresis (12% SDS-PAGE). As a negative control, native proteins (without linker modifications) were incubated with the fluorophores under the same experimental conditions.

Protein−DNA Conjugation. *(a) Through Michael Addition.* 10 nmol of shD1 [100 μM stock solution in ddH_2O] or shD2 were incubated with 60 μmol of DTT [1 M] for 2 h at RT to allow for reduction of disulfide bridges, with small molecules removed by gel filtration through NAP5 and NAP10 columns and eluted in 1× PBS buffer (pH = 7.4). These solutions were mixed with 60 nmol of freshly prepared BL-1 protein conjugate solutions [80 μM], incubated overnight at 4 °C in dark, filtered using Vivaspin filtation system (STV: cutoff 10 kDa; Mb: cutoff 5 kDa) and concentrated to a final volume of 1 mL (in 20 mM Tris buffer pH = 8.3). The conjugates were purified by ion-exchange chromatography by using a Mono Q 5/50 column.

(b) Through Cu Catalyzed Huisgen Cycloaddition. 100 nmol of $CuSO_4$ was mixed with 5 μmol of sodium ascorbate. A color change from light blue to light brown could be observed and the Cu^{1+} ions were stabilized by the addition of 0.5 μmol Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA). The solution was mixed with 10 nmol of alkD1 or alkD2, incubated for 5 min on ice, added to 60 nmol freshly prepared BL-2 modified protein solutions, filled up to a final volume of 1 mL with 1× PBS (pH = 7.4) and incubated overnight at 4 °C in the dark. The final concentrations were 100 μM $CuSO_4$, 500 μM THPTA, 5 mM sodium ascorbate, and 10 μM Alkyne ssDNA. Reaction mixture was filtered with Vivaspin filtration column (STV: cutoff 10 kDa; Mb: cutoff 5 kDa), rebuffered in 20 mM Tris (pH = 8.3) and purified by ion-exchange chromatography using a Mono Q 5/50.

(c) Through Cu-Free Huisgen Cycloaddition. 60 nmol of freshly prepared BL-3 protein conjugates were mixed with 10 nmol of the corresponding azide modified ssDNA azD1 or azD2 and incubated overnight at 4 °C in the dark. Reaction mixture was then filtered though Vivaspins (STV: cutoff 10 kDa; Mb: cutoff 5 kDa) and the buffer was exchanged to 20 mM Tris (pH = 8.3). The purification was completed by ion exchange liquid chromatography.

Characterization of DNA−Protein Conjugates. The concentrations of all fractions were determined by UV−vis spectroscopy and the main fractions were characterized by native PAGE.

Activity Study of DNA-Mb Conjugates. Peroxidase activity of ^{Mb}**BL-1**$_{D1}$, ^{Mb}**BL-1**$_{D2}$, ^{Mb}**BL-3**$_{D1}$ and ^{Mb}**BL-3**$_{D2}$ and native Mb were measured with a Synergy multiwellplate reader (Biotek) and were carried out in black 96 well microtiterplates.

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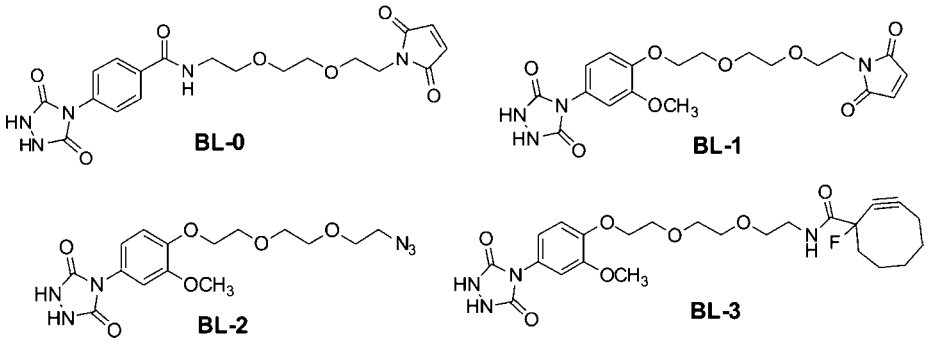

Figure 1. Structure of the tyrosine binding bifunctional linkers for the attachment of ssDNA through Michael addition (**BL-0** and **BL-1**), copper(I)-catalyzed (**BL-2**), and copper-free (**BL-3**) 1,3-Huisgen addition.

To 1 pmol of protein conjugates and Mb [50 μL, 20 nM solution in KP_i pH 6 buffer pH 6] 50 μL of AmplexRed dye solution (prepared according to manufacturers instructions, Invitrogen) and H_2O_2 were added. Final concentrations of all reagents in 100 μL volume were: 50 μM AmplexRed, 1 mM H_2O_2, and 10 nM of protein. Each measurement was done in triplicate over a time period of 1 h by using λ_{ex} at 530 nm (λ_{em} at 590 nm). The sensitivity setting at the Synergy plate reader was set to 100.

DNA Directed Immobilization (DDI) of DNA-Mb Conjugates. DDI of ^{Mb}**BL-1**$_{D1}$, ^{Mb}**BL-1**$_{D2}$, ^{Mb}**BL-3**$_{D1}$, ^{Mb}**BL-3**$_{D2}$, and **native Mb** as a negative control was carried out in black, STV coated 96-well microtiterplates (MTPs) prepared according to published protocols.[28] The freshly prepared STV coated MTPs were incubated with 50 μL complementary biotinylated strands bcD1 and bcD2 in TETBS buffer [240 nM, 1.2 nmol] for 45 min at RT. Subsequently, the wells were washed four times with 200 μL TETBS buffer followed by incubation for 10 min with TETBS-biotin buffer [20 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.05% Tween, 800 μM biotin]. The plates were washed again two times in 200 μL TETBS and were incubated with 50 μL ^{Mb}**Y**$_D$ conjugates [20 nM] in KP_i buffer (pH = 6.0) for 1 h at RT in the dark. After incubation, wells were washed four times with 200 μL KP_i buffer (pH = 6.0) and filled up with 50 μL KP_i to enable the study of protein activity. The AmplexRed/H_2O_2 reaction solution in KP_i buffer (pH = 6.0) was freshly prepared according to manufacturer's instructions and 50 μL aliquots were added to each well. Measurements were done in triplicate over 1 h using λ_{ex} at 530 nm (λ_{em} at 590 nm).

RESULTS AND DISCUSSION

Bifunctional Linker Preparation and Activation. Our concept makes use of the methodology previously described by Ban et al. where tyrosine binding cyclic diazodicarboxamide derivatives were used for fluorescent labeling of peptides and model proteins.[29] Previously, modification of Tyr was achieved through use of cyclic imines[30] or diazonium salt,[31] but the use of diazocarboxamide is particularly promising as it is fast, selective and allows introduction of different functional groups. We have based the design of our linker on diazocarboxamide functionality and introduced maleimide (BL-1), azide (BL-2), and cyclooctyne (BL-3) groups (Figure 1) to afford clickable bifunctional linkers and to enable coupling of DNA onto the tyrosine residues of two model proteins, streptavidin (STV) and horse heart myoglobin (Mb). Both proteins found numerous applications in bio- and nanotechnology either as anchoring elements (both STV[32] and STV-DNA conjugates[7,33]) or as excellent models for the study of heme enzymes, in particular, heme cofactor reconstitution (Mb).[34,35] STV−DNA conjugates have also extensively been used for DNA nanostructuring and heme containing pseudoperoxidase Mb was interesting for us since we are currently looking into methodologies for the synthesis of Mb-DNA conjugates without the involvement of Cys, Lys, or cofactor to enable multimodal modification on the way toward the artificial protein−DNA machine. From a structural point of view, STV contains 24 Tyr in 4 protein subunits and Mb only 2 Tyr, one positioned close to the C terminus (Tyr146) and away from the cofactor catalytic site, while the second one is closer to the active pocket (Tyr103). This allows us to study the tyrosine binding i.e. to explore how many different conjugates are obtained upon the DNA binding or how the tyrosine number affects the efficiency of the conjugation and the protein's native function. This is particularly interesting in the case of Mb, which has been previously shown to have enhanced peroxidase activity upon modification with short DNA strands.[18,20]

We first attempted to use a similar linker, which described by Ban et al. (Figure 1, **BL-0**) for conjugation of DNA through Michael addition; however, the modification of both STV and Mb was not successful in our hands. After considering the proposed mechanism of reaction (SI, Figure S3C), we reasoned that the electron donating groups positioned on the benzyl ring might improve the linker's stability in aqueous solution and therefore result in desired products. Therefore, we have designed new linkers **BL-1**, **BL-2** and **BL-3** in such a way to contain methoxy benzyl diazocarboxamide, short ethylene glycol chain for improved solubility and the clickable moieties to enable DNA attachment through Michael addition (maleimide), Cu(I) catalyzed (azide) and copper free (cyclooctyne) Huisgen 1,3-dipolar cycladdition (Figure 1). Tyrosine reactivity of prepared linkers was first explored by direct reaction with free amino acid. The oxidation of the linkers to the corresponding diazocarboxamides was achieved by treatment with N-bromosuccinimide (NBS) and pyridine (Pyr) in DMF and the rapid change of color indicated immediate formation of activated diazocarboxamides (SI, Figure S3B), which were then coupled to the tyrosine. Mass spectrometry showed that, in the case of the above linkers, desired tyrosine coupling products are obtained, and therefore, the linkers could be employed further for protein coupling.

Flourescent Labeling Using Bifunctional Linkers. Prior to DNA modification, we explored tyrosine coupling by employing the bifunctional linkers for fluorescent labeling of the proteins using appropriate dyes, *S*-acetylmercapto-succinoyl-amino-fluorescein (SAMSA) for Michael addition, cyclo-

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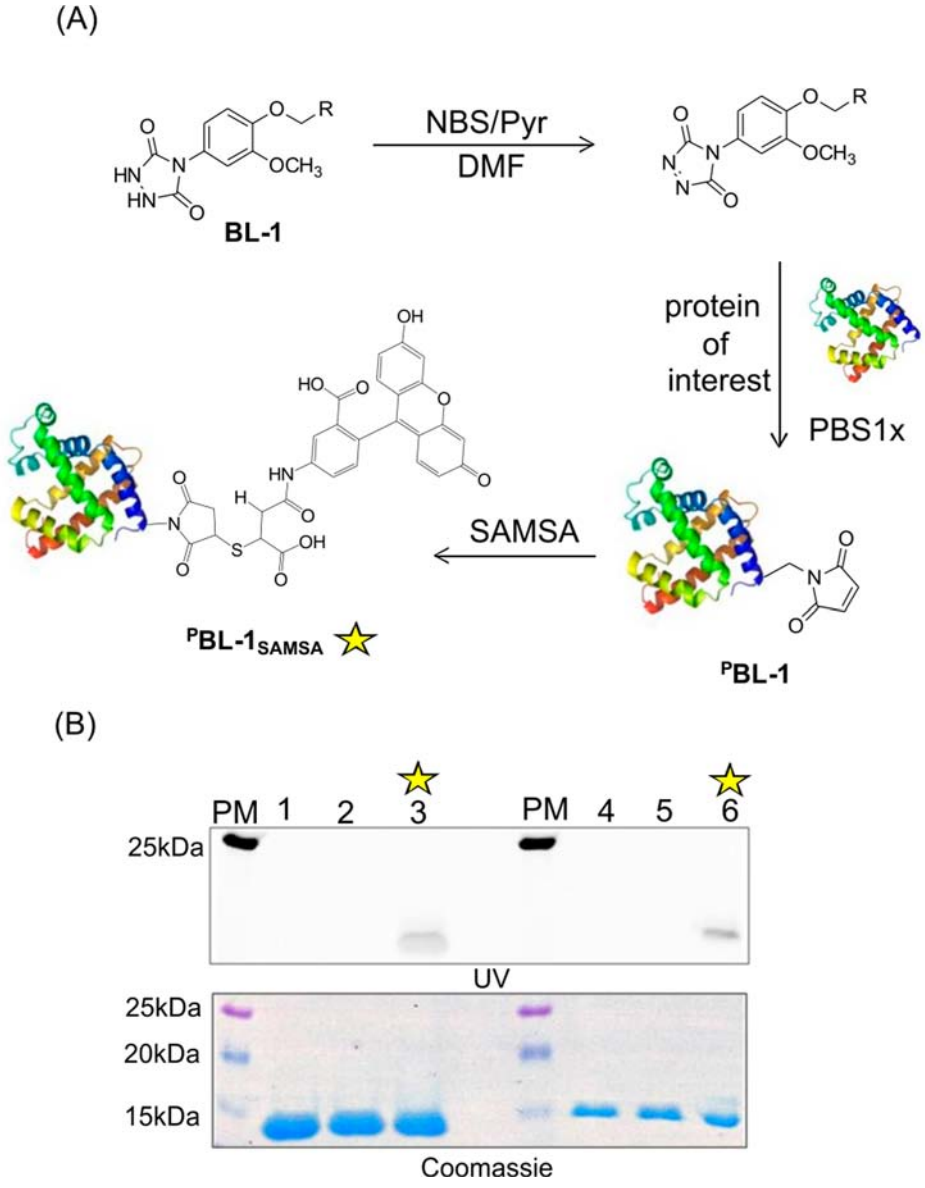

Figure 2. (A) Coupling of fluorescent dye (SAMSA) to the protein surface through the bifunctional linker **BL-1** to afford fluorescently labeled ^{P}**BL-1**$_{SAMSA}$ conjugates. (B) SDS-PAGE gel of ^{STV}**BL-1**$_{SAMSA}$ (lanes 1−3) and ^{Mb}**BL-1**$_{SAMSA}$ (lanes 4−6) conjugates. **PM**: protein marker; **1**: native STV; **2**: STV negative control (native STV incubation with SAMSA); **3**: ^{STV}**BL-1**$_{SAMSA}$; **4**: native Mb; **5**: Mb negative control (native Mb incubation with SAMSA); **6**: ^{Mb}**BL-1**$_{SAMSA}$. The gel was first characterized under UV irradiation followed by protein staining with Coomassie blue.

octyne-fluorescein (COF) and the azide-modified fluorophore (AzF) for Huisgen cycloaddition. The representative example of fluorescence labeling through Michael addition is shown in Figure 2. Linkers were first oxidized with NBS and Pyr in DMF and are allowed to react with the protein in PBS buffer (DMF content ca. 5−10%) for 15 min at r.t. At this point, it is important to note that the amount of NBS and NBS-Pyr ratio need to be carefully controlled, as we have observed that higher ratios (and higher NBS amount) can lead to protein damage (SI, Figure S1 and S2). After removal of excess linker and reagents, modified proteins were incubated with a 10-fold excess of the appropriate dyes and left to react overnight. For clarity reasons, we have decided to use ^{P}**Y**$_{F}$ nomenclature for obtained conjugates, where **P** represents protein, **F** the fluorophore, and **Y** the linker used. As can be seen in Figure 2 B, only linker modified proteins containing maleimide group reacted with SAMSA (lane 3, STV and lane 6 Mb). The same was true for other linkers and corresponding dyes, whereby it has to be mentioned that ^{Mb}**BL-2**$_{COF}$ conjugate demonstrated only low band intensity most probably due to the instability of used COF dye (SI Figures S4−S5). Negative controls in which native proteins were incubated with corresponding dyes under the same experimental conditions showed no fluorescence, indicating high specificity of the reaction. Fluorophore coupling was additionally confirmed by UV−vis spectroscopy by monitoring both protein and fluorophore absorption (SI, Figures S6−S8).

DNA Protein Conjugation. Successful coupling of the fluorophores using different click methodologies convinced us to proceed to the next step of DNA coupling. Two different 22-mer ssDNA (**D1** and **D2**; see SI for sequences) with appropriate 5′-end modification (thiol, azide or alkyne) were used to prepare corresponding protein conjugates ^{P}**Y**$_{D}$ using NBS/Pyr activation step (15 min) and subsequent DNA

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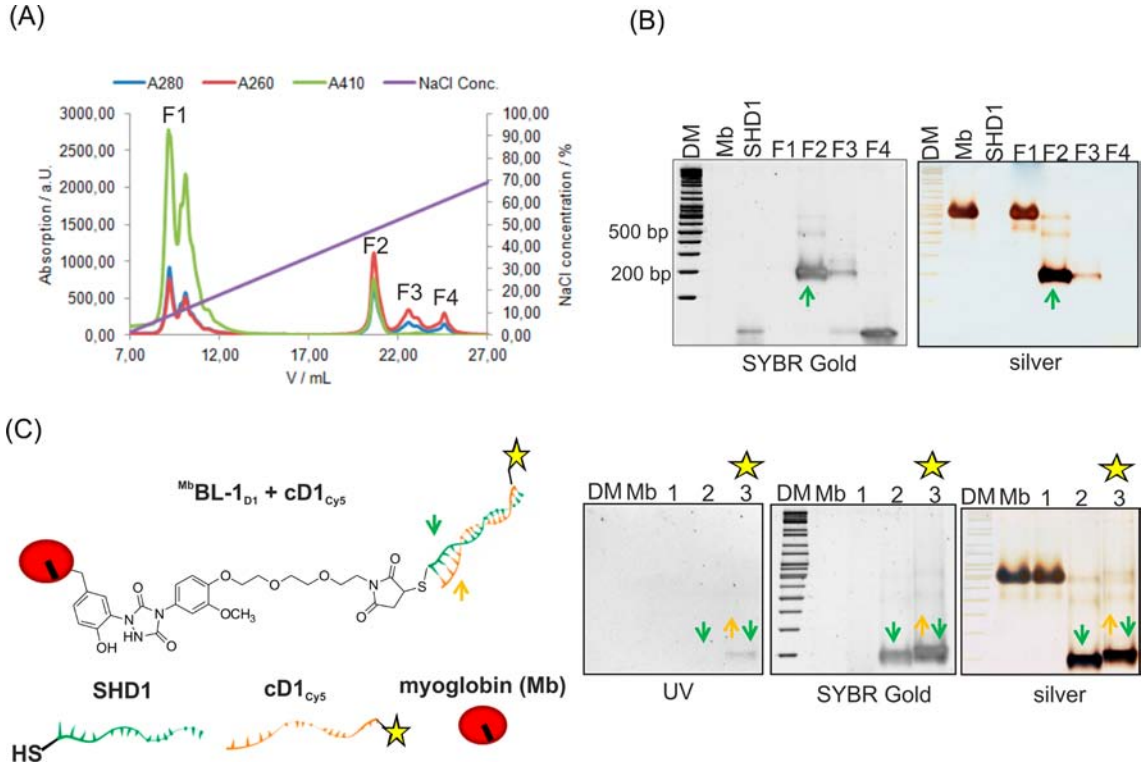

Figure 3. (A) FPLC chromatogram of ^{Mb}**BL-1**$_{D1}$ conjugate purification. (B) Native PAGE characterization of the purified FPLC fractions F1, F2, F3, and F4. Staining procedure: (I) SYBR gold; (II) silver staining. (C) Structure of the ^{Mb}**BL-1**$_{D1}$ conjugate and native PAGE characterization of the hybridization reaction with complementary **cD1**$_{Cy5}$ (orange arrow). **DM**: 10 kbp DNA-Marker; Mb: native myoglobin; **1**: negative control (native **Mb** + **D1** and **cD1**$_{Cy5}$); **2**: ^{Mb}**BL-1**$_{D1}$ conjugate; **3**: ^{Mb}**BL-1**$_{D1}$ + **cD1**$_{Cy5}$. Staining procedure: (I) UV irradiation; (II) SYBR Gold; (III) silver staining.

coupling with or without the addition of Cu catalyst in the case of Huisgen cycloaddition. For DNA coupling, a 6-fold excess of freshly prepared, tyrosine modified proteins ^{P}**Y** were mixed with corresponding ssDNA and incubated overnight. The conjugates were then purified using fast protein liquid chromatography (FPLC) with ion exchange column and additionally characterized by UV−vis spectroscopy and gel electrophoresis, in which PAGE native gels were first stained by SYBR Gold to indicate the presence of ssDNA followed by silver staining for protein identification. Shown in Figure 3 is the preparation and characterization of ^{Mb}**BL-1**$_{D1}$ conjugate. Several signals can be observed in FPLC spectra (Figure 3A, F1, F2, F3, F4) and the gel electrophoresis (Figure 3B) confirmed that F1 contained native Mb, F2 the desired conjugate, and fractions F3 and F4 unreacted DNA. As seen in Figure 3B, fraction F2 shows higher mobility than native Mb due to the increase in the amount of negative charges upon the DNA conjugation to the protein.

To test if the hybridization ability of ssDNA is still preserved after the conjugation, we have incubated Cy5-modified complementary DNA strands, **cD1**$_{Cy5}$ and **cD2**$_{Cy5}$ (25 equiv, over 1 h at r.t), with corresponding conjugates. As shown in Figure 3C, ^{Mb}**BL-1**$_{D1}$ conjugate can be hybridized, indicated by the shift in the protein band and the successful detection of Cy5 fluorescence after UV irradiation (lane 3). Negative controls in which native proteins were mixed with fluorescent complementary **cD2** showed no bands. To calculate the number of ssDNA strands per Mb, UV−vis spectroscopy was used and the obtained Mb/D1 ratio was 1:1, indicating the presence of 1 strand per Mb and modification of single Tyr. However, it should be noted that two additional, lower intensity bands can be observed in the fractions containing the ^{Mb}**Y**$_{D}$ conjugates (Figure 3B, fraction F2; and SI Figures S9, S14, S15), both with larger shifts but much lower yields (ca. 6% and 1% compared to the main band). They might represent the bi-DNA-Mb and tri-DNA-Mb conjugates—they contain DNA that can hybridize with **cD1**$_{Cy5}$ as indicated by the band shifts upon the addition of complementary sequence (Figure 3C, lane 2 and 3; and SI Figure S18, S21). Biconjugation is the result of the modification of both Tyr residues present in Mb, while triconjugation indicated that there is a possible, low yielding, reaction with another amino acids present in the protein. Ban et al. reported that there is a small amount of side product obtained probably due to the largely nonfavored reaction of the tyrosine binding linker with tryptophan or lysine.[29]

Conjugates ^{Mb}**BL-1**$_{D2}$, ^{STV}**BL-1**$_{D1}$, and ^{STV}**BL-1**$_{D2}$ were also successfully prepared by varying the type of DNA proving that the conjugation strategy is not sequence dependent (SI, Figure S9−S11, SI pg 22 for the overall yields of reactions, which are up to 15% and comparable to the standard DNA−protein coupling through Lys modifying sulfo-SMCC linker). It should be noted that STV contains more Tyr residues than Mb, and as can be seen from FPLC spectra, besides the monoconjugate, which is obtained in the highest amount (94%, fraction F2, SI, Figure S10−S11), a mixture of higher conjugates is also obtained (fraction F4, SI, Figure S10−S11). The high amount of monoconjugates could be explained by the use of higher amount of protein relative to DNA and the short reaction time for protein−linker modification. In the case of copper free 1,3-Huisgen addition using cyclooctyne linker BL-3, all four conjugates (^{Mb}**BL-3**$_{D1}$, ^{Mb}**BL-3**$_{D2}$,^{STV}**BL-3**$_{D1}$, and ^{STV}**BL-3**$_{D2}$) were successfully prepared and characterized (SI, Figures S14−

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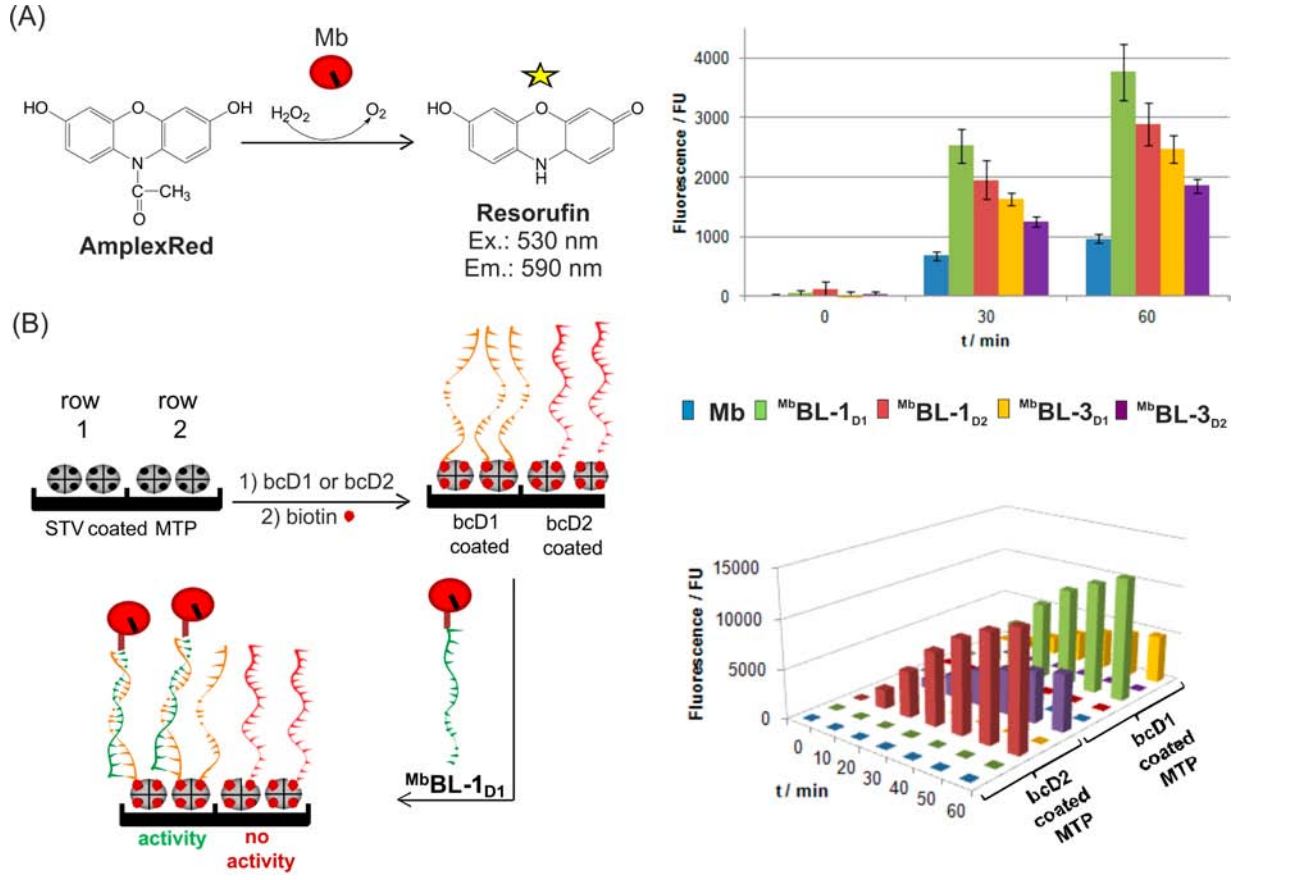

Figure 4. (A) Peroxidase activity assay using AmplexRed dye and the activity of prepared conjugates in comparison to native Mb (blue). 1 pmol of conjugates was used in the assay. (B) Scheme of the DNA-directed immobilization (DDI) of ^{Mb}**BL-1$_D$** conjugates on STV-coated microtiterplates (MTPs) and the results of the activity study of the immobilized conjugates. **cD1** and **cD2** were used as capture oligos.

S17 and S21−S22,). However, Cu-catalyzed conjugation of alkyne-DNA onto the Mb containing azide group introduced via BL-2 linker was not successful in our hands. We hypothesize that the reason might be the presence of the heme pocket containing Fe^{2+} core and a possible coordination of the azide linker with the heme cofactor, which might influence the stability and reactivity of both diazodicarboxamide-NBS intermediate (SI, Figure S3) and the azide group. This hypothesis was further confirmed by successful preparation of the DNA conjugates with azide modified, nonheme protein STV (^{STV}**BL-2$_{D1}$** and ^{STV}**BL-2$_{D2}$**, SI, Figures S12−S13 and S20). Further experiments concerning heme enzymes and Cu(I)-catalyzed reactions are ongoing and will be described elsewhere.

Activity of DNA-Mb Conjugates. Next, we were interested to see if the binding of the DNA through the tyrosine affects the inherent pseudoperoxidase activity of Mb. To achieve that, we have employed an assay in which the oxidation of nonfluorescent dye (AmplexRed) into fluorescent resorufin is achieved in the presence of H_2O_2 and peroxidase enzymes. As shown in Figure 4A, Mb-DNA conjugates remain fully active upon DNA conjugation. In fact, the activity of prepared Mb conjugates is significantly higher than that of the native Mb.

Furthermore, the ^{Mb}**Y$_{D1}$** conjugates showed higher activity than the ^{Mb}**Y$_{D2}$** conjugates, whereas the ^{Mb}**BL-1$_D$** conjugates have higher peroxidase activity (up to 4-fold increase for ^{Mb}**BL-1$_{D1}$** and 3-fold increase for ^{Mb}**BL-1$_{D2}$**) than the ^{Mb}**BL-3$_D$** conjugates (around 2-fold increase for both ^{Mb}**BL-3^{D1}** and ^{Mb}**BL-3$_{D2}$**) in comparison to native Mb. It was reported previously that the activity of Mb can increase upon the DNA conjugation through cofactor[18] and that the sequence and the length of the DNA can have a marked effect on the peroxidase activity of the conjugates due to the possible interactions of the charged DNA groups with the protein surface and the stabilization of the protein's active pocket.[36] The difference in activity between the protein modified through maleimide (**BL-1**) and cyclooctyne (**BL-3**) can be explained by the possible steric hindrance or the other types of interaction of cyclooctyne-azide ring with the protein surface, which might affect the accessibility of the heme pocket (which is in the case of Mb positioned close to the surface of the protein). Nevertheless, the activity of both conjugates is preserved and increased, which indicates the high potential of tyrosine modification for DNA-conjugate preparation. In fact, when compared to the conjugates prepared using sulfo-SMCC bifunctional linker and Lys modification, Mb-DNA shows only up to 2-fold increase (for **D1**) and 1.5-fold increase (for **D2**) of activity in comparison to native Mb (SI, Figure S26).

DNA Directed Immobilization of DNA-Mb Conjugates. Finally, to demonstrate the applicability of tyrosine modification strategy to prepare functional protein−DNA conjugates for further use in DNA nanostructuring and DNA directed immobilization, Mb-DNA conjugates were immobilized onto solid surface using biotinylated capture complementary DNA sequences **bcD1** and **bcD2** (Figure 4B). The peroxidase activity of the ^{Mb}**Y$_D$** conjugates was measured after

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the immobilization and showed the same pattern as the solution experiments. Negative controls containing no DNA and noncomplementary strands showed no activity, which indicated the specificity of the DNA sequence controlled protein immobilization through Watson Crick base pairing.

CONCLUSIONS

In conclusion, we have synthesized tyrosine binding bifunctional clickable linker to prepare DNA−protein conjugates using simple two-step procedure and click chemistry methodologies. Marked increase in Mb activity compared to the native protein was observed and the conjugates successfully immobilized using DNA directed immobilization. We believe that these linkers will find an application not only in design of novel and multi-DNA−protein conjugates, but will also be useful for more complex designs of artificial enzymes and for further advance of DNA nanostructuring and origami decoration.

ASSOCIATED CONTENT

Supporting Information

A detailed description of the synthesis of bifunctional linkers, characterizations of protein coupling with corresponding fluorophores, purifications and PAGE characterizations of all successful $^{Y}\mathbf{P}_{D}$ conjugates as well as negative controls, activity tests and absorption spectra after fluorophore coupling. This material is available free of charge via the Internet at http://pubs.acs.org

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Rothemund, P. W. (2006) Folding DNA to create nanoscale shapes and patterns. *Nature 440*, 297−302.
(2) Seeman, N. C. (2003) DNA in a material world. *Nature 421*, 427−31.
(3) Seeman, N. C. (2003) Biochemistry and structural DNA nanotechnology: an evolving symbiotic relationship. *Biochemistry 42*, 7259−69.
(4) Niemeyer, C. M., and Ceyhan, B. (2001) DNA-directed functionalization of colloidal gold with proteins. *Angew. Chem., Int. Ed. 40*, 3685−3688.
(5) Adler, M., Wacker, R., and Niemeyer, C. M. (2003) A real-time immuno-PCR assay for routine ultrasensitive quantification of proteins. *Biochem. Biophys. Res. Commun. 308*, 240−50.
(6) Boozer, C., Ladd, J., Chen, S., and Jiang, S. (2006) DNA-directed protein immobilization for simultaneous detection of multiple analytes by surface plasmon resonance biosensor. *Anal. Chem. 78*, 1515−9.
(7) Bano, F., Fruk, L., Sanavio, B., Glettenberg, M., Casalis, L., Niemeyer, C. M., and Scoles, G. (2009) Toward multiprotein nanoarrays using nanografting and DNA directed immobilization of proteins. *Nano Lett. 9*, 2614−8.
(8) Hazarika, P., Ceyhan, B., and Niemeyer, C. M. (2005) Sensitive detection of proteins using difunctional DNA-gold nanoparticles. *Small 1*, 844−8.
(9) Numajiri, K., Yamazaki, T., Kimura, M., Kuzuya, and A. Komiyama, M. (2010) Discrete and active nzyme nanoarrays on DNA origami scaffolds purified by affinity tag separation. *J. Am. Chem. Soc. 132*, 9937−9.
(10) Bier, F. F., Kleinjung, F., Ehrentreich-Forster, E., and Scheller, F. W. (1999) Changing functionality of surfaces by directed self-assembly using oligonucleotides-the Oligo-Tag. *Biotechniques 27*, 752−756 , 758, 760.
(11) Niemeyer, C. M., Sano, T., Smith, C. L., and Cantor, C. R. (1994) Oligonucleotide-directed self-assembly of proteins: semi-synthetic DNA–streptavidin hybrid molecules as connectors for the generation of macroscopic arrays and the construction of supra-molecular bioconjugates. *Nucleic Acids Res. 22*, 5530−9.
(12) Sacca, B., Meyer, R., Erkelenz, M., Kiko, K., Arndt, A., Schroeder, H., Rabe, K. S., and Niemeyer, C. M. (2010) Orthogonal protein decoration of DNA origami. *Angew. Chem., Int. Ed. 49*, 9378−83.
(13) Meyer, R., and Niemeyer, C. M. (2011) Orthogonal protein decoration of DNA nanostructures. *Small 7*, 3211−8.
(14) Niemeyer, C. M. (2010) Semisynthetic DNA-protein conjugates for biosensing and nanofabrication. *Angew. Chem., Int. Ed. 49*, 1200−16.
(15) Braun, G., Diechtierow, M., Wilkinson, S., Schmidt, F., Huben, M., Weinhold, E., and Reich, N. O. (2008) Enzyme-directed positioning of nanoparticles on large DNA templates. *Bioconjugate Chem. 19*, 476−9.
(16) Goodman, R. P., Erben, C. M., Malo, J., Ho, W. M., McKee, M. L., Kapanidis, A. N., and Turberfield, A. J. (2009) A facile method for reversibly linking a recombinant protein to DNA. *ChemBioChem 10*, 1551−7.
(17) Fruk, L., Kuo, C. H., Torres, E., and Niemeyer, C. M. (2009) Apoenzyme reconstitution as a chemical tool for structural enzymology and biotechnology. *Angew. Chem., Int. Ed. 48*, 1550−74.
(18) Fruk, L., and Niemeyer, C. M. (2005) Covalent hemin-DNA adducts for generating a novel class of artificial heme enzymes. *Angew. Chem., Int. Ed. 44*, 2603−6.
(19) Simon, P., Dueymes, C., Fontecave, M., and Decout, J. L. (2005) DNA detection through signal amplification by using NADH: flavin oxidoreductase and oligonucleotide-flavin conjugates as cofactors. *Angew. Chem., Int. Ed. 44*, 2764−7.
(20) Fruk, L., Muller, J., and Niemeyer, C. M. (2006) Kinetic analysis of semisynthetic peroxidase enzymes containing a covalent DNA-heme adduct as the cofactor. *Chem.—Eur. J. 12*, 7448−57.
(21) Lovrinovic, M., Fruk, L., Schroder, H., and Niemeyer, C. M. (2007) Site-specific labeling of DNA-protein conjugates by means of expressed protein ligation. *Chem. Commun.*, 353−5.
(22) Duckworth, B. P., Chen, Y., Wollack, J. W., Sham, Y., Mueller, J. D., Taton, T. A., and Distefano, M. D. (2007) A universal method for the preparation of covalent protein-DNA conjugates for use in creating protein nanostructures. *Angew. Chem., Int. Ed. 46*, 8819−22.
(23) Chandra, R. A., Douglas, E. S., Mathies, R. A., Bertozzi, C. R., and Francis, M. B. (2006) Programmable cell adhesion encoded by DNA hybridization. *Angew. Chem., Int. Ed. 45*, 896−901.
(24) Dey, S., and Sheppard, T. L. (2001) Ketone-DNA: a versatile postsynthetic DNA decoration platform. *Org. Lett. 3*, 3983−6.
(25) Tominaga, J., Kemori, Y., Tanaka, Y., Maruyama, T., Kamiya, N., and Goto, M. (2007) An enzymatic method for site-specific labeling of recombinant proteins with oligonucleotides. *Chem. Commun.*, 401−3.
(26) Jongsma, M. A., and Litjens, R. H. (2006) Self-assembling protein arrays on DNA chips by auto-labeling fusion proteins with a single DNA address. *Proteomics 6*, 2650−5.
(27) Lee, H. S., Dimla, R. D., and Schultz, P. G. (2009) Protein-DNA photo-crosslinking with a genetically encoded benzophenone-containing amino acid. *Bioorg. Med. Chem. Lett. 19*, 5222−4.

(28) Niemeyer, C. M., Wacker, R., and Adler, M. (2003) Combination of DNA-directed immobilization and immuno-PCR: very sensitive antigen detection by means of self-assembled DNA-protein conjugates. *Nucleic Acids Res. 31*, e90.
(29) Ban, H., Gavrilyuk, J., and Barbas, C. F., 3rd (2010) Tyrosine bioconjugation through aqueous ene-type reactions: a click-like reaction for tyrosine. *J. Am. Chem. Soc. 132*, 1523−5.
(30) Guo, H. M., Minakawa, M., Ueno, L., and Tanaka, F. (2009) Synthesis and evaluation of a cyclic imine derivative conjugated to a fluorescent molecule for labeling of proteins. *Bioorg. Med. Chem. Lett. 19*, 1210−3.
(31) Gavrilyuk, J., Ban, H., Nagano, M., Hakamata, W., and Barbas, C. F., 3rd (2012) Formylbenzene diazonium hexafluorophosphate reagent for tyrosine-selective modification of proteins and the introduction of a bioorthogonal aldehyde. *Bioconjugate Chem. 23*, 2321−8.
(32) Reznik, G. O., Vajda, S., Cantor, C. R., and Sano, T. (2001) A streptavidin mutant useful for directed immobilization on solid surfaces. *Bioconjugate Chem. 12*, 1000−4.
(33) Niemeyer, C. M., Koehler, J., and Wuerdemann, C. (2002) DNA-directed assembly of bienzymic complexes from in vivo biotinylated NAD(P)H:FMN oxidoreductase and luciferase. *ChemBioChem 3*, 242−5.
(34) Roncone, R., Monzani, E., Murtas, M., Battaini, G., Pennati, A., Sanangelantoni, A. M., Zuccotti, S., Bolognesi, M., and Casella, L. (2004) Engineering peroxidase activity in myoglobin: the haem cavity structure and peroxide activation in the T67R/S92D mutant and its derivative reconstituted with protohaemin-l-histidine. *Biochem. J. 377*, 717−24.
(35) Sato, H., Hayashi, T., Ando, T., Hisaeda, Y., Ueno, T., and Watanabe, Y. (2004) Hybridization of modified-heme reconstitution and distal histidine mutation to functionalize sperm whale myoglobin. *J. Am. Chem. Soc. 126*, 436−7.
(36) Glettenberg, M., and Niemeyer, C. M. (2009) Tuning of peroxidase activity by covalently tethered DNA oligonucleotides. *Bioconjugate Chem. 20*, 969−75.