DNA–Enzyme Conjugates

DOI: 10.1002/anie.201206962

Enhancement and Modulation of Enzymatic Activity through Higher-Order Structural Changes of Giant DNA–Protein Multibranch Conjugates**

*Sergii Rudiuk, Anna Venancio-Marques, and Damien Baigl**

Conjugating an oligonucleotide to a protein is a widely used strategy to combine the protein function with the recognition ability provided by the short DNA fragment. On the other hand, genomic DNA molecules, owing to their extremely large size, have the unique ability to undergo dramatic higher-order structural changes upon addition of compaction/unfolding agents, but the influence of these structural changes on a conjugated protein has never been explored. Herein, we describe the first preparation of giant DNA–protein multibranch conjugates and study how regulated higher-order structural changes of DNA can control the function of the protein. These conjugates are composed of a β-lactamase enzyme attached to one to four 48.5 kbp lambda phage DNA (λ DNA) "branches". We show that the conjugation of giant DNA increases the enzymatic activity, which can be decreased by compaction with spermine and recovered by unfolding with NaCl.

Conjugating DNA to a protein is a common strategy in modern biotechnology. It is however usually done with DNA in the form of short oligonucleotides (up to several tens of bases), which are used to recognize complementary nucleic acid targets. Such protein–oligonucleotide conjugates have led to many biosensing applications as probes for the presence of nucleic acids or proteins[1,2] and to the preparation of protein microarrays[2,3] or protein complexes[4] by DNA-mediated assembly. Additionally, DNA scaffolds with well-defined 2D and 3D nanostructures, for instance made by using DNA origami,[5] have been used to precisely organize conjugated proteins at the nanometer scale.[6,7] In this approach, the DNA scaffold is usually made of a highly ordered DNA structure with characteristic dimensions up to a few hundred nanometers. However, to our knowledge, the behavior of proteins, and more specifically of enzymes, conjugated to giant (greater than 10 kbp) duplex DNA molecules has never been investigated. We prepared for the first time giant DNA–protein hybrid conjugates where a β-lactamase enzyme was included in the center of a branched complex composed of up to four λ DNA (48.5 kbp) molecules. We studied the activity of the enzyme inside these conjugates. Since giant DNA molecules can undergo a dramatic and reversible higher-order structural transition between an unfolded, elongated coil state and a very dense, compact state,[8–10] we investigated how the enzymatic activity was regulated through such higher-order structural changes.

First, we studied the possibility of attaching a relatively short duplex DNA to β-lactamase using the streptavidin–biotin interaction.[11,12] This was done by assembling a streptavidin–β-lactamase conjugate (S-βLac) with a monobiotinylated 541 bp DNA (B-DNA541, Figure 1a). S-βLac had a hydrodynamic diameter of about 6 nm according to DLS measurements (Supporting Information, Figure S1) while B-DNA541 was expected to have a diameter of 2 nm and a contour length of about 184 nm. Atomic force microscopy (AFM) observations after deposition on mica revealed the presence of mainly four kinds of structures composed of a higher central part surrounded by one to four branches with a length of 173 ± 9 nm (Figure 1a, right panels). The central part and branches were thus attributed to S-βLac and conjugated DNA molecules, respectively. We also observed a few other structures with a larger number (5–14) of branches (Figure S2), which could be attributed to the presence of aggregates or streptavidin/β-lactamase ratios different than 1:1 in the starting material. Our results show that the streptavidin–biotin interaction allows for the attachment of one to four duplex DNAs to S-βLac, in agreement with previously reported work using unconjugated streptavidin.[13]

Next, we applied a similar strategy with biotinylated λ DNA instead of B-DNA541. In this case, we could not observe any branched structures, neither by AFM nor by fluorescence microscopy. The difficulty to directly conjugate several biotinylated λ DNAs to S-βLac could be attributed to steric hindrance or electrostatic repulsion between the giant λ DNA molecules. We thus devised a two-step conjugation method (Figure 1b). First, a short, 12 nt biotinylated oligonucleotide (B-oligo) complementary to one of the sticky ends of λ DNA was attached to S-βLac. The resulting conjugates (βLac-oligo) were then mixed with a fourfold excess of

[*] Dr. S. Rudiuk, A. Venancio-Marques, Prof. D. Baigl
Department of Chemistry, Ecole Normale Supérieure
24 rue Lhomond, 75005 Paris (France)
and
Université Pierre et Marie Curie Paris 6
75005 Paris (France)
and
UMR 8640, CNRS (France)
E-mail: damien.baigl@ens.fr
Homepage: http://www.baigllab.com/

[**] We thank G. Hallais and Dr. A. Gautier for experimental support and discussion. The research leading to these results received funding from the European Research Council (ERC) [European Community's Seventh Framework Programme (FP7/2007-2013)/ERC Grant agreement no. 258782] and from the Institut Universitaire de France (IUF).

Supporting information for this article (experimental details) is available on the WWW under http://dx.doi.org/10.1002/anie.201206962.

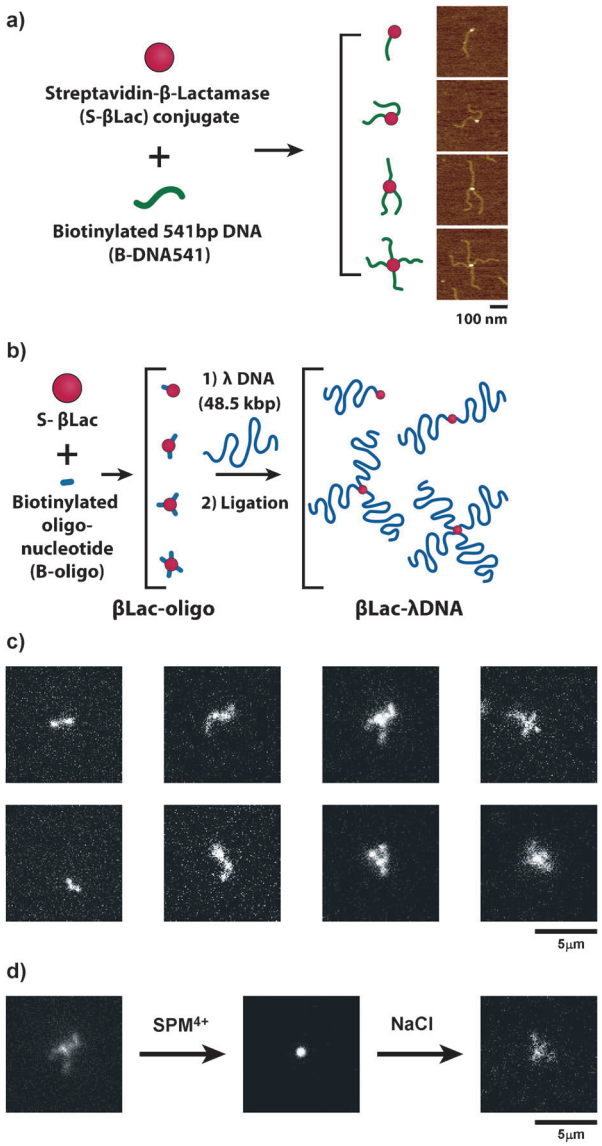

Figure 1. Preparation and observation of DNA–protein multibranch conjugates. a) Biotinylated 541 bp DNA (B-DNA541) was bound to a streptavidin–β-lactamase conjugate (S-βLac) to form complexes with S-βLac surrounded by 1–4 DNA branches, as shown by AFM on mica (right). b) A biotinylated 12 nt oligonucleotide (B-oligo) was conjugated to S-βLac to form βLac-oligo complexes with 1–4 oligonucleotide branches. λ DNA (48.5 kbp) was then ligated to the oligonucleotides to give four conjugates (βLac-λDNA) with 1–4 giant DNA branches. c) From left to right, two representative fluorescence microscopy (FM) images for each of the four structures in the βLac-λDNA solution. [DNA] = 2.5 μM (of nucleotides) in 10 mM Tris-HCl buffer (pH 7.4); [YOYO-1] = 0.01 μM. d) Representative FM images of βLac-λDNA alone (left), compacted by spermine (middle), and decompacted after addition of NaCl (right). Same conditions as (c) with $[SPM^{4+}] = 100$ μM; [NaCl] = 100 mM.

λ DNA prior to a ligation reaction with T4 DNA ligase to potentially give four kinds of β-lactamase/λ DNA complexes (βLac-λDNA; Figure 1b, right). The resulting mixture was characterized by fluorescence microscopy (FM), which is a particularly suitable technique to observe individual giant DNA molecules in solution.[14] We observed highly fluctuating coil-like structures, which could be categorized into four main types. The first type consisted of a fluctuating coil (Movie S1 and Figure 1c, left) with an average apparent size of $l \approx 1.5 \pm 0.2$ μm, which is very similar to the value reported for single molecules of λ DNA in solution.[15] This structure could thus be unbound λ DNA or S-βLac conjugated to a single λ DNA. The three other types consisted of larger structures with two (Movie S2 and Figure 1c, middle left), three (Movie S3 and Figure 1c, middle right), or four (Movie S4 and Figure 1c, right) DNA branches. Moreover, these complexes had an average apparent size $l \approx 3.0 \pm 0.3$ μm. The size and appearance of these complexes were therefore in agreement with the four putative structures of βLac-λDNA shown in Figure 1b, but the highly dynamic nature of these conjugates made it hard to quantitate their distribution. To our knowledge, this was the first time that such giant DNA–protein multibranch conjugates had been prepared. Interestingly, the addition of the tetravalent cationic DNA compaction agent spermine ($[SPM^{4+}] = 100$ μM) induced an abrupt change in the appearance of βLac-λDNA. In this case, all conjugates appeared as bright spots with a high diffusion coefficient, which can be attributed to a much smaller particles (Movie S5 and Figure 1d, middle). We observed an apparent size of about $l \approx 0.6 \pm 0.2$ μm, which, because of the blurring effect in fluorescence images, was probably larger than the real size.[16] We thus concluded that βLac-λDNA underwent compaction upon addition of spermine, similar to the compaction of linear giant DNA molecules by polyamines.[10,17]

It is known that the addition of monovalent salts can induce the unfolding of DNA previously compacted by a polyamine through an ion-exchange mechanism.[10,18,19] We therefore studied the effect of adding NaCl (100 mM) to βLac-λDNA compacted by spermine (100 μM). Under these conditions, images had very low contrast, which is typical of fluorescence microscopy observations in the presence of high salt concentrations. We could however distinguish fluctuating structures, which looked similar to βLac-λDNA before compaction (Movie S6 and Figure 1d, right). The size was estimated to be $l \approx 2.7 \pm 0.2$ μm, which is comparable to that of βLac-λDNA before compaction. These results show that βLac-λDNA multibranch conjugates undergo compaction by spermine followed by unfolding by NaCl.

We then studied the effect of λ DNA conjugation on the enzymatic activity of β-lactamase. For this purpose, we used nitrocefin (NC), a chromogenic substrate that undergoes a β-lactam ring opening reaction from a yellow form ($\lambda_{max} \approx 390$ nm) to a red product ($\lambda_{max} \approx 490$ nm) upon conversion by β-lactamase (Figure S3).[20–22] Figure 2a shows the absorbance of the red product as a function of time after nitrocefin addition. In the absence of enzyme, nitrocefin (NC alone) slowly underwent hydrolysis. In contrast, when β-lactamase was added to the solution, the absorbance increased significantly, which indicates that the enzymatic reaction had occurred. Interestingly, the kinetics of the reaction were strongly depended on the nature of β-lactamase conjugation. Although S-βLac (solution 1) and βLac-oligo (solution 2) displayed similar kinetics, βLac-λDNA (solution 4) showed much faster nitrocefin conversion. A control experiment where S-βLac was mixed with the same amount of λ DNA in

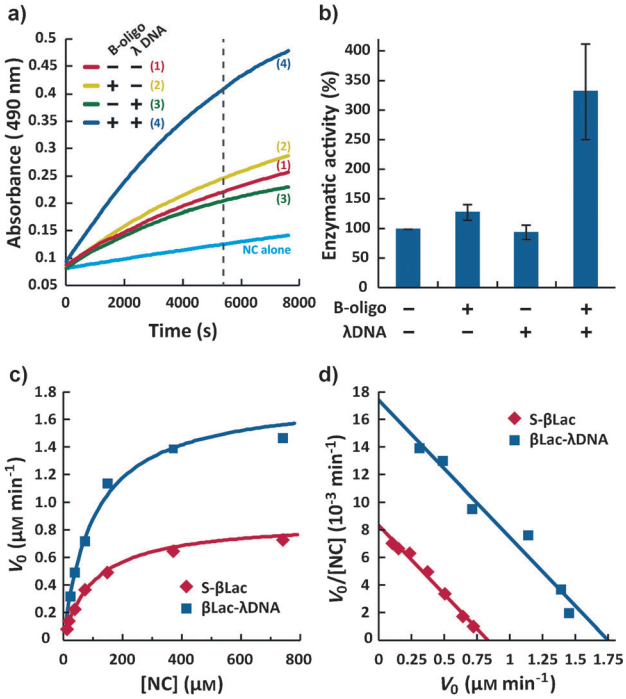

Figure 2. Measurement of enzymatic activity of β-lactamase using a chromogenic nitrocefin (NC) substrate. a) Absorbance measured at 490 nm as a function of time. [NC] = 75 μM. b) Enzymatic activity [Eq. (1)] of solutions (1)–(4) measured after 90 min of nitrocefin conversion (dashed line in (a)). Error bars show mean values ± SD from triplicate samples. c) The initial conversion rate (V_0) as a function of nitrocefin concentration for S-βLac and βLac-λDNA. Solid lines correspond to least-squares fitting curves according to Michaelis–Menten kinetics: $V_0 = V_{max}[NC]/(K_M+[NC])$ where V_{max} = maximum initial rate and K_M = Michaelis constant. d) Eadie–Hofstee plot of data shown in (c). Solid lines are linear fits where the slope = $-1/K_M$ and x-intercept = V_{max}. For all experiments: [β-lactamase] = 114 pM; [Tris-HCl] = 40 mM (pH 7.4); T = 30 °C.

the absence of B-oligo (solution 3) displayed a kinetics similar to that of S-βLac alone. To quantify these differences, we calculated the enzymatic activity (EA) using Equation (1):

$$EA\ (\%) = (A_{sample} - A_{NC})/(A_{ref} - A_{NC}) \times 100 \quad (1)$$

where A_{sample}, A_{NC}, and A_{ref} are the absorbance at 490 nm after 90 minutes (dashed line in Figure 2a) of the sample of interest, nitrocefin alone, and S-βLac (without spermine or NaCl) used as a reference. Figure 2b shows EA values for different β-lactamase complexes. Strikingly, it shows that the EA for βLac-λDNA (+B-oligo, +λ DNA) was about 3.3-fold higher than that of S-βLac (−B-oligo, −λ DNA). In the presence of the oligonucleotide alone (+B-oligo, −λ DNA), a slight enhancement was observed (EA = 129 ± 13 %) but it was much smaller than that of βLac-λDNA (EA = 332 ± 81 %). This shows that the presence of giant DNA molecules was necessary to induce the increase in the enzymatic activity. Moreover, when λ DNA was present in the solution but not attached to S-βLac (−B-oligo, +λ DNA), the enzymatic activity (EA = 95 ± 12 %) was very similar to that of S-βLac alone. This demonstrates that the physical linkage between the enzyme and giant DNA was crucial for the enhancement of the enzymatic activity.

To gain more insight into the mechanism at the origin of this enhancement, we measured the initial conversion rate V_0 as a function of nitrocefin substrate concentration ([NC]) at a given enzyme concentration (114 pM) for S-βLac and βLac-λDNA. Fitting the data from Figure 2c showed that both S-βLac and βLac-λDNA followed Michaelis–Menten kinetics. These data also established that, regardless of NC concentration, V_0 was larger in the case of βLac-λDNA than in the case of S-βLac. Interestingly, the Eadie–Hofstee plot (Figure 2d) not only confirmed that the maximal rate V_{max} (x-intercept) was larger for βLac-λDNA than for S-βLac, but also showed that the Michaelis constant K_M (−1/slope) was similar for both enzymes. Table 1 gives the parameters K_M, k_{cat} (V_{max}/[β-lactamase]), and k_{cat}/K_M obtained by fitting data (as shown in Figure 2c) in triplicate. It confirms that both S-βLac and βLac-λDNA had a similar K_M, which shows that the presence of giant DNA did not significantly affect the affinity of the β-lactamase for nitrocefin. In contrast, both k_{cat} and k_{cat}/K_M displayed a two-fold increase in the case of βLac-λDNA. This shows that the conjugation to giant DNA enhanced the enzymatic activity mainly by increasing the catalytic activity of the enzyme, rather than by modifying the affinity of the enzyme for the substrate. It has been previously reported that the activity of an enzyme can be affected by the presence of a conjugated oligonucleotide.[23] Our results show that the enzymatic activity of β-lactamase is increased by incorporating the enzyme into a micro-environment composed of giant DNA molecules.

Table 1: Kinetic data determined by Michaelis–Menten analysis of S-βLac and βLac-λDNA as shown in Figure 2c.[a]

	K_M [μM]	k_{cat} [×10^3 min^{-1}]	k_{cat}/K_M [μM^{-1} min^{-1}]
S-βLac	80 ± 12	5.7 ± 1.2	71 ± 8.8
βLac-λDNA	96 ± 9.2	12 ± 2.7	123 ± 38

[a] Values are mean values ± SD from triplicate samples.

Figure 1d showed that higher-order structures of βLac-λDNA multibranch conjugates could be controlled, using spermine to induce the compaction of the DNA branches and NaCl to unfold them after compaction. We thus studied how enzymatic activity was affected by DNA compaction. Figure 3a shows the EA of S-βLac and βLac-λDNA as a function of spermine (SPM^{4+}) concentration. The enzymatic activity of S-βLac alone was almost independent of SPM^{4+} for $0 \le [SPM^{4+}] \le 150$ μM. In contrast, a distinct effect of $[SPM^{4+}]$ was observed in the case of βLac-λDNA conjugate. For $0 \le [SPM^{4+}] \le 25$ μM, EA was high (around 350 %), which confirmed the enhancement of enzymatic activity of β-lactamase conjugated to giant DNA. At $[SPM^{4+}] = 50$ μM, a marked drop in EA was observed (EA = 388 ± 79 % for $[SPM^{4+}] = 25$ μM and 135 ± 41 % for $[SPM^{4+}] = 50$ μM). For higher SPM^{4+} concentrations, EA became similar to that of S-βLac alone. Since enzymatic activity assays were performed at a high DNA concentration (44 μM nucleotides), fluorescence microscopy could not be used to follow βLac-λDNA com-

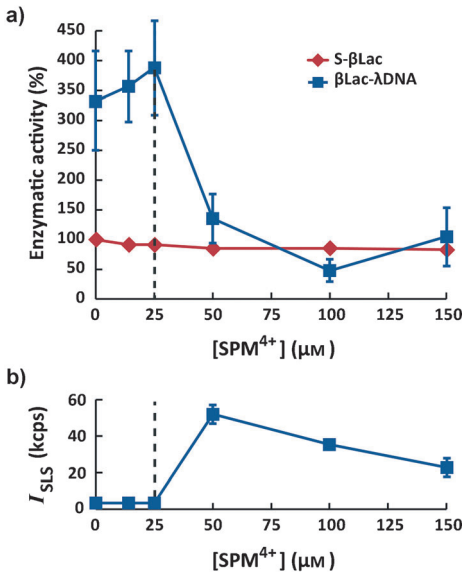

Figure 3. Effect of spermine (SPM^{4+}) on the enzymatic activity and DNA compaction. a) Enzymatic activity [Eq. (1)] as a function of spermine concentration for S-βLac and βLac-λDNA. [β-lactamase] = 114 pM; [NC] = 75 μM; [Tris-HCl] = 40 mM (pH 7.4); T = 30 °C. b) λ DNA compaction followed by static light scattering intensity (I_{SLS}) as a function of spermine concentration. [λ DNA] = 44 μM (of nucleotides); [Tris-HCl] = 40 mM (pH 7.4); T = 30 °C. Symbols and error bars show mean values ± SD from triplicate samples. Error bars smaller than the symbols are not shown.

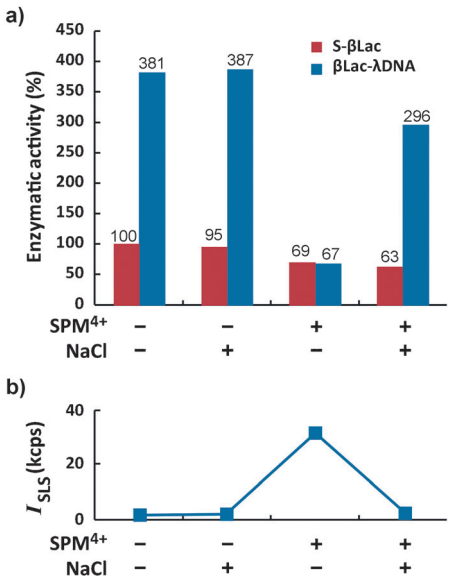

Figure 4. Effect of spermine and NaCl on enzymatic activity and DNA compaction/unfolding. a) Enzymatic activity [Eq. (1)] in the presence or absence of spermine (100 μM) and NaCl (100 mM), for S-βLac alone and βLac-λDNA. [β-lactamase] = 114 pM; [NC] = 75 μM; [Tris-HCl] = 40 mM (pH 7.4); T = 30 °C. b) λ DNA compaction followed by static light scattering intensity (I_{SLS}) in the presence or absence of spermine (100 μM) and NaCl (100 mM). [λ DNA] = 44 μM (of nucleotides); [Tris-HCl] = 40 mM (pH 7.4); T = 30 °C.

paction/unfolding. We therefore used static light scattering (SLS) to monitor the compaction state of λ DNA under the conditions of the EA measurements. Interestingly, the SLS intensity (I_{SLS}) as a function of [SPM^{4+}] (Figure 3b) correlated well with the change in EA (Figure 3a). For $0 \le [SPM^{4+}] \le 25$ μM, I_{SLS} was low, which is a typical feature of unfolded DNA.[18,24] In contrast, for $50 \le [SPM^{4+}] \le 150$ μM, a strong increase in I_{SLS} was observed, which is a signature of DNA compaction.[18,24] The dashed line shown in Figure 3a and b thus corresponds to the onset of λ DNA compaction. These results show that the enzymatic activity of βLac-λDNA is enhanced when the protein–DNA complex is in an unfolded state while it decreases to a level similar to that of unconjugated S-βLac when the complex has been compacted by spermine.

We then studied the effect of unfolding the compacted DNA using NaCl. Figure 4a shows the enzymatic activity of S-βLac and βLac-λDNA measured in the presence or absence of spermine (100 μM) and NaCl (100 mM). In the absence of spermine, the EA was not affected by the presence of NaCl for both samples. The addition of spermine in the absence of NaCl resulted, as shown before, in a strong decrease in the EA of βLac-λDNA, close to the value of S-βLac alone. Interestingly, when both spermine and NaCl were present in the solution, we observed a partial recovery of EA for βLac-λDNA while the value of S-βLac alone was not significantly affected. Moreover, preliminary Michaelis–Menten analysis showed that the k_{cat}/K_M decreased in the presence of spermine and increased again after the addition of NaCl (Table S1). To correlate the change in EA for βLac-λDNA with its λ DNA compaction state, we measured the I_{SLS} of λ DNA under the conditions of the EA measurements. Figure 4b shows that the λ DNA remained in an unfolded state upon addition of NaCl, was compacted by the addition of SPM^{4+}, and was unfolded again when NaCl was added after compaction by SPM^{4+}. This is further evidence of the correlation between the enzymatic activity and the compaction state of βLac-λDNA. The enhanced enzymatic activity, after decreasing from compaction of βLac-λDNA by spermine, can be partially recovered by unfolding the complex with NaCl.

In summary, we have described the first preparation of multibranch conjugates made of a single protein connected to several giant DNA molecules. These conjugates are composed of a β-lactamase enzyme conjugated to between one and four λ DNA branches. Single-molecule observations showed that these conjugates could be folded and unfolded upon addition of tetravalent spermine and monovalent NaCl, respectively. We found that the enzymatic activity of β-lactamase was strongly affected by the micro-environment produced by the giant DNA. When the DNA was unfolded, the enzymatic activity was increased. Interestingly, this enhancement could be decreased and then recovered by DNA compaction and unfolding, respectively. Similar results were observed for another giant DNA–protein multibranch conjugate where the central protein was the β-galactosidase enzyme (Figure S4). All of these results show that conjugating giant DNA to a protein can strongly influence its activity and enables modulation of activity through higher-order structural changes of the giant DNA. We demonstrated this with a simple stimulation based on the addition of monovalent and multivalent salts, but it can be extended to other stimuli such

as light,[25–27] pH,[28] redox reaction,[29] or temperature.[30] Conversely, the conjugated protein could also be used as a reporter of DNA higher-order structural changes in response to biochemical or biophysical stimulations.

Received: August 28, 2012
Revised: October 2, 2012
Published online: November 9, 2012

Keywords: conjugates · DNA · DNA compaction · enzymes · enzymatic activity

[1] C. M. Niemeyer, *Angew. Chem.* **2010**, *122*, 1220 – 1238; *Angew. Chem. Int. Ed.* **2010**, *49*, 1200 – 1216.
[2] B. Saccà, C. M. Niemeyer, *Chem. Soc. Rev.* **2011**, *40*, 5910 – 5921.
[3] C. M. Niemeyer, L. Boldt, B. Ceyhan, D. Blohm, *Anal. Biochem.* **1999**, *268*, 54 – 63.
[4] M. Erkelenz, C.-H. Kuo, C. M. Niemeyer, *J. Am. Chem. Soc.* **2011**, *133*, 16111 – 16118.
[5] P. W. K. Rothemund, *Nature* **2006**, *440*, 297 – 302.
[6] J. Fu, M. Liu, Y. Liu, N. W. Woodbury, H. Yan, *J. Am. Chem. Soc.* **2012**, *134*, 5516 – 5519.
[7] B. Saccà, C. M. Niemeyer, *Angew. Chem.* **2012**, *124*, 60 – 69; *Angew. Chem. Int. Ed.* **2012**, *51*, 58 – 66.
[8] V. A. Bloomfield, *Curr. Opin. Struct. Biol.* **1996**, *6*, 334 – 341.
[9] V. A. Bloomfield, *Biopolymers* **1997**, *44*, 269 – 282.
[10] A. Estévez-Torres, D. Baigl, *Soft Matter* **2011**, *7*, 6746 – 6756.
[11] T. Sano, C. Smith, C. Cantor, *Science* **1992**, *258*, 120 – 122.
[12] C. M. Niemeyer, M. Adler, B. Pignataro, S. Lenhert, S. Gao, L. Chi, H. Fuchs, D. Blohm, *Nucleic Acids Res.* **1999**, *27*, 4553 – 4561.
[13] Y. Tian, Y. He, A. E. Ribbe, C. Mao, *Org. Biomol. Chem.* **2006**, *4*, 3404 – 3405.
[14] K. Yoshikawa, M. Takahashi, V. Vasilevskaya, A. Khokhlov, *Phys. Rev. Lett.* **1996**, *76*, 3029 – 3031.
[15] H. Oana, K. Tsumoto, Y. Yoshikawa, K. Yoshikawa, *FEBS Lett.* **2002**, *530*, 143 – 146.
[16] K. Yoshikawa, Y. Matsuzawa, *Physica D* **1995**, *84*, 220 – 227.
[17] L. C. Gosule, J. A. Schellman, *Nature* **1976**, *259*, 333 – 335.
[18] R. W. Wilson, V. A. Bloomfield, *Biochemistry* **1979**, *18*, 2192 – 2196.
[19] L. Cinque, Y. Ghomchi, Y. Chen, A. Bensimon, D. Baigl, *ChemBioChem* **2010**, *11*, 340 – 343.
[20] A. Venancio-Marques, Y.-J. Liu, A. Diguet, T. di Maio, A. Gautier, D. Baigl, *ACS Synth. Biol.* **2012**, DOI: 10.1021/sb300010a.
[21] C. H. O'Callaghan, A. Morris, S. M. Kirby, A. H. Shingler, *Antimicrob. Agents Chemother.* **1972**, *1*, 283 – 288.
[22] D. M. Livermore, *Clin. Microbiol. Rev.* **1995**, *8*, 557 – 584.
[23] M. Glettenberg, C. M. Niemeyer, *Bioconjugate Chem.* **2009**, *20*, 969 – 975.
[24] V. Vijayanathan, T. Thomas, A. Shirahata, T. J. Thomas, *Biochemistry* **2001**, *40*, 13644 – 13651.
[25] A.-L. M. Le Ny, C. T. Lee, *J. Am. Chem. Soc.* **2006**, *128*, 6400 – 6408.
[26] A. Estévez-Torres, C. Crozatier, A. Diguet, T. Hara, H. Saito, K. Yoshikawa, D. Baigl, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 12219 – 12223.
[27] A. Diguet, N. K. Mani, M. Geoffroy, M. Sollogoub, D. Baigl, *Chem. Eur. J.* **2010**, *16*, 11890 – 11896.
[28] N. Makita, K. Yoshikawa, *Biophys. Chem.* **2002**, *99*, 43 – 53.
[29] Y. Yamasaki, K. Yoshikawa, *J. Am. Chem. Soc.* **1997**, *119*, 10573 – 10578.
[30] D. Matulis, I. Rouzina, V. A. Bloomfield, *J. Mol. Biol.* **2000**, *296*, 1053 – 1063.

