ARTICLE

OBC
www.rsc.org/obc

Synthesis of fluorescent oligonucleotide–EYFP conjugate: Towards supramolecular construction of semisynthetic biomolecular antennae

Florian Kukolka and Christof M. Niemeyer*
Universität Dortmund, Fachbereich Chemie Biologisch-Chemische Mikrostrukturtechnik, Otto-Hahn-Str. 6, D-44227 Dortmund Germany. E-mail: cmn@chemie.uni-dortmund.de; Fax: + 49 (0)231/755 7082

Received 4th May 2004, Accepted 17th June 2004
First published as an Advance Article on the web 8th July 2004

A novel species of DNA–protein conjugate was synthesized by chemically linking DNA oligonucleotides to *Aequorea victoria* green fluorescent protein mutant EYFP. An additional cysteine was added to the C-terminus of the EYFP by genetic engineering and used to covalently attach amino-modified oligonucleotide with the aid of the heterobifunctional crosslinker sSMCC. EYFP maintained its fluorescence upon conjugation. The oligonucleotide provides an additional binding site to the fluorescent protein, and hence, the EYFP conjugate could be specifically hybridized with both complementary DNA–protein conjugates in-solution as well as immobilized at capture oligonucleotides attached to a solid substrate. These studies are paving the way for future applications in the self-assembly of photoactive supramolecular complexes, such as artificial light-harvesting systems.

Introduction

There is currently great interest in the synthesis of artificial light harvesting devices from multiple chromophoric units in order to study the fundamental principles of photosynthesis as well as to develop advanced materials and devices with complex spectroscopic properties. Recent attempts to synthesize light harvesting complexes through self-assembly included the use of porphyrin derivatives,[1–3] polypyridine complexes of d^6 metal ions[4,5] and perylene based polyphenyl dendrimers.[6–9] On the other hand, many researcher groups are currently exploiting the large variety of biomolecules, in particular proteins and nucleic acids, as building blocks for the assembly of functional devices.[10] In this context, the bioconjugation of short DNA oligonucleotides to various molecular compounds and materials has already extensively been used to construct nanoscaled functional devices.[10–12] As an example, the extraordinary specific molecular recognition of complementary ssDNA-sequences has been utilized for the spatially defined immobilization of semisynthetic DNA–protein conjugates, both at the micrometer and nanometer length scale.[10,13,14]

We here report initial results on the development of DNA–protein conjugates for the supramolecular construction of semisynthetic biomolecular antennae. To this end, a 24mer ssDNA oligomer was chemically conjugated to the enhanced yellow fluorescent protein (EYFP), a mutant of naturally occuring *Aequorea victoria* green fluorescent protein, revealing red shifted absorption and emission maxima.[15] This kind of fluorescent proteins are often used as markers in life sciences and as components of fluorescence energy transfer (FRET) in the study of molecular interactions.[16] We intend to use the DNA-conjugated EYFP as a molecular building block in the DNA-directed assembly of new types of nanoscaled optical devices, which operate by FRET mechanisms as optical switches or supramolecular light harvesting complexes and antennae systems. Binding studies revealed that the oligonucleotide of the conjugate can be used as a recognition site for the selective hybridization to complementary nucleic acids both in homogeneous solutions and in solid-phase hybridization assays.

Results and discussion

The use of the heterobifunctional crosslinker sulfosuccinimidyl 4-[*N*-maleimidomethyl]-cyclohexane-1-carboxylate (sSMCC) in the synthesis of DNA–protein conjugates has proven to be very effective.[14] With this crosslinker, two principal coupling strategies can be used. Firstly, aminogroups of lysine residues within the protein can be reacted with the sSMCC to introduce maleimido groups and then the protein is coupled with DNA containing thiol groups.[14] Conversely, amino-modified DNA oligomers can be activated with the sSMCC and are subsequently coupled with proteins containing exposed thiol groups, such as available from cysteine residues (Fig. 1). The latter coupling strategy should enable a site-specific attachment since proteins usually contain various amino groups while thiol groups are less abundant and can be selectively incorporated at distinguished sites by mutagenesis.

The EYFP contains 20 lysine-groups while only two cysteins are present which are too far apart from each other to form an

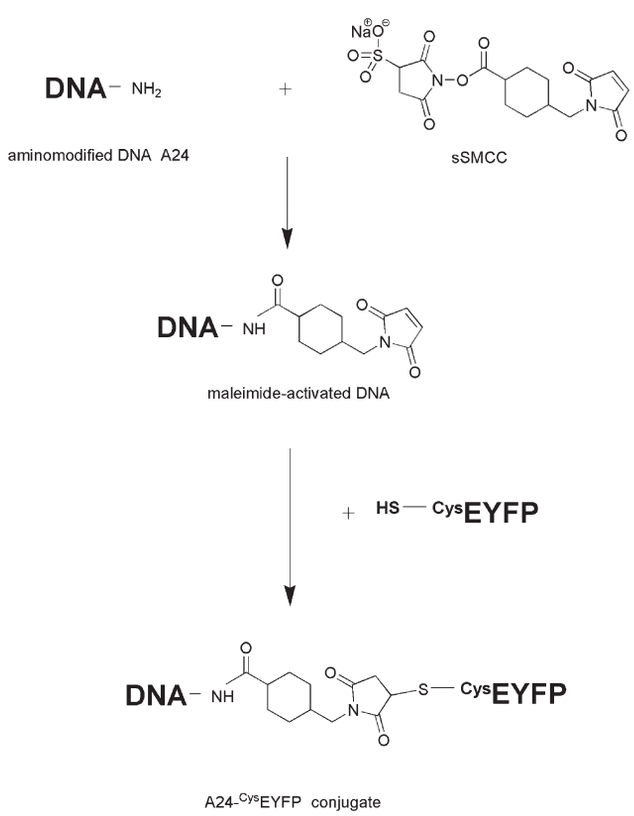

Fig. 1 Schematic drawing of the crosslinking of DNA with ^{Cys}EYFP by maleimide derivatization of aminomodified DNA A24 and subsequent reaction with the thiol group of the protein.

DOI: 10.1039/b406492e

intramolecular disulfide bond. Moreover, one cysteine is known to be buried inside of the protein while the other one should be available for coupling, as revealed by crystallographic analysis.[15] Hence, we initially attempted to couple the 24mer amino-modified oligonucleotide A24 with either one of the two cysteins available in native EYFP. Essentially no conjugate formation was observed, indicating that the two cysteins are not accessible for DNA-coupling (data not shown). We then attempted the inversed strategy by coupling the thiolated DNA oligomer with the EYFP's lysine amino groups. This led to extensive protein aggregation resulting in a clogging of the column during the purification subsequent to EYFP activation with sSMCC. Likely, the sSMCC-induced aggregation was due to *in situ* cross-reaction of the maleimide derivatized protein with the protein's cystein groups (or *vice versa*). Since the addition of maleimide activated DNA did not yield any conjugates, one might speculate about differences in sterical and/or electrostatic properties between the protein-protein and the DNA–protein interaction, respectively.

To prevent protein-protein crosslinking, we attempted to protect the thiol groups of the EYFP with 5,5′-dithio-bis-[2-nitrobenzoic acid] (DTNB)[17] prior to the reaction with the crosslinker. Although it had previously been reported that low concentrations of 1 mM DTNB irreversibly bleach GFP,[18] in our experiments EYFP fluorescence was not affected by DTNB even at concentrations of 7.5 mM (data not shown). This protection step, in fact, allowed the synthesis of conjugates, however, unusually broad conjugate peaks were obtained during the purification by anion-exchange chromatography (data not shown). As shown in lane 2 of Fig. 2, the SDS-PAGE analysis of this experiment revealed multiple conjugate bands. Analysis of the EYFP reacted with sSMCC without the addition of amino-DNA also showed several products with a major band at about twice the size of the EYFP at a molecular weight of *ca.* 31 kDA (lane 1). These results suggested the occurrence of cross-linking, which led to the formation of a series of adducts with varying DNA:protein stoichiometries during the DNA conjugation. Our observation that the several adducts formed were not separated by chromatography might also be due to the intrinsic tendency of EYFP to form dimers. According to the manufacturer's instructions (Clontech), *Aequorea victoria* fluorescent proteins, such as EYFP, dimerize *via* hydrophobic interactions upon increasing ionic strength, as encountered here during purification. This fact is also illustrated in lane 3 of Fig. 2, where a weak band at about 60 kDa possibly indicates dimerization of EYFP. Small amounts of dimers might have been re-formed or were not fully dissociated during sample preparation. Otherwise, small amounts of covalent conjugates might have been formed, *e.g.* by reductive coupling *via* cysteine thiol groups.

To overcome these problems we designed an EYFP-mutant by adding a cysteine residue at the C-terminus of EYFP through genetic engineering, using PCR with appropriately modified primers.

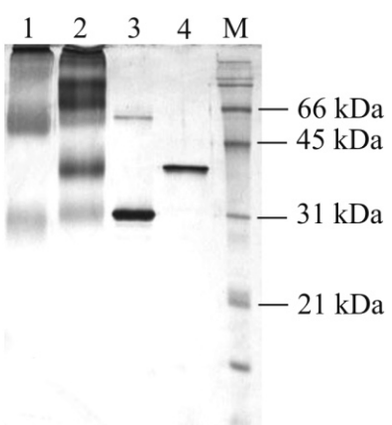

Fig. 2 Analysis of the conjugate synthesis. This is a denaturing 10% SDS-PAGE, stained by silver development. Lane 1: sSMCC-activated EYFP; lane 2: conjugates obtained from the coupling of thiolated DNA A24 with EYFP; lane 3: native EYFP, note the band of the dimer; lane 4: purified A24-^{Cys}EYFP conjugate, M: Bio-Rad broad-range molecular weight marker.

The PCR-product was then cloned into the vector pRSET-A (Invitrogen), which adds the codons for a N-terminal polyhistidine tag (6xHis-tag) to the inserted gene, enabling for easy purification through affinity chromatography of the expressed protein. The resulting plasmid was used to transform *E. coli* BL21 and the protein was overexpressed after induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Up to 30 mg/l of the recombinant ^{Cys}EYFP were obtained, which could readily be purified by Nickel–Nitrilotriacetic Acid (Ni–NTA) chromatography taking advantage of the 6xHis-tag present in the ^{Cys}EYFP. In contrast to the numerous coupling products formed in the above described cross linking reactions, the conjugation of the ^{Cys}EYFP showed the expected elution profile with clearly separated peaks and basically no reaction side-products (Fig. 3). The resulting conjugate A24-^{Cys}EYFP (Peak III, Fig. 3) was eluted at about 450 mM NaCl and appeared as a single peak in between the peaks of the unreacted protein (Peak I and II) and DNA (Peak IV). The DNA-^{Cys}EYFP conjugate and the corresponding reactants were identified by both native and denaturing PAGE, as shown in Figs. 4 and 5 (Lanes 1–6), respectively.

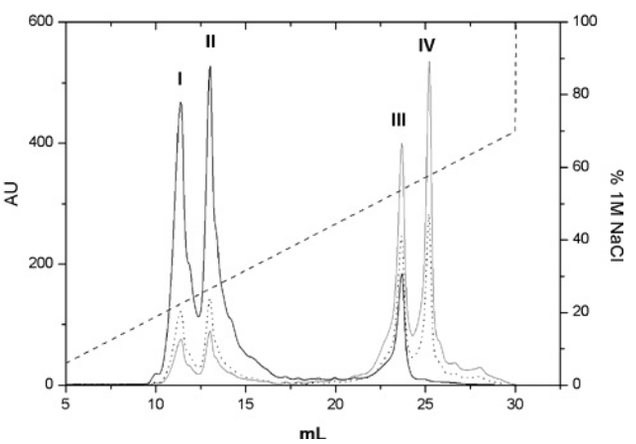

Fig. 3 Purification of A24-^{Cys}EYFP conjugate by ion-exchange chromatography. Shown are the absorbance at 515 nm (black), the absorbance at 280 nm (dotted) and 260 nm (grey) as well as the gradient of the NaCl concentration (dashed). The peaks are numbered in roman numbers in order of their elution.

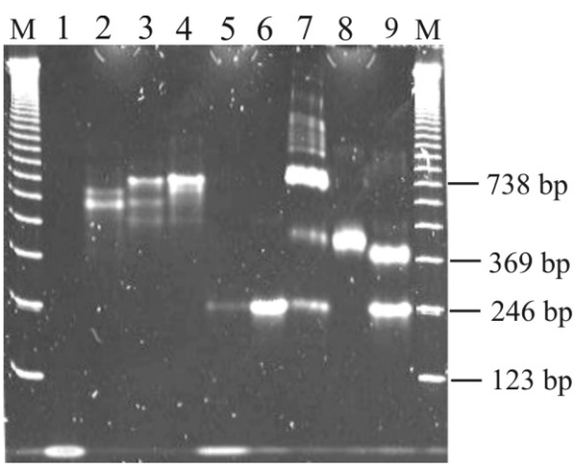

Fig. 4 Analysis of conjugate purification and gel-shift experiment. This is a 10% non-denaturing PAGE stained with SybrGold. The sample peaks I–IV correspond to the peaks from the ion-exchange purification in Fig. 3. Lane 1: sSMCC activated A24, lane 2: DTT activated ^{Cys}EYFP, lane 3: Peak I, lane 4: Peak II, lane 5: Peak IV, lane 6: Peak III, lane 7: A24-^{Cys}EYFP hybridized with complementary cA24-STV, lane 8: cA24-STV, lane 9: Hybridization of A24-^{Cys}EYFP with non-complementary A24-STV as negative control, M: 123bp ladder.

During chromatographic purification, fractions containing the fluorescent ^{Cys}EYFP were identified by monitoring the absorbance at its absorption maximum of 515 nm. The relatively weak absorbance of the ^{Cys}EYFP conjugate at 515 nm during the purification is due to the quenching of EYFP fluorescence by the increased

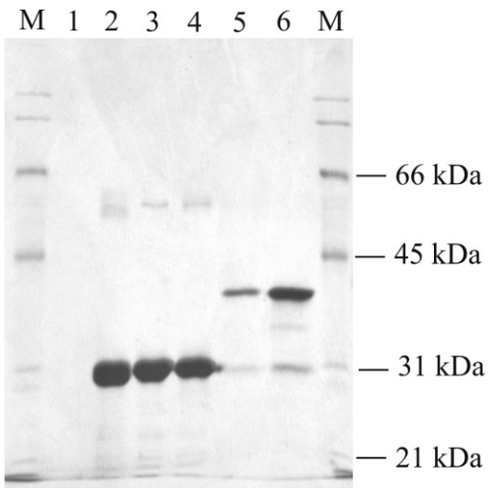

Fig. 5 Analysis of conjugate purification. This is a denaturing 10% SDS-PAGE stained by silver development. The sample peaks I–IV correspond to the peaks from the ion-exchange purification in Fig. 3. Lane 1: sSMCC activated A24, lane 2: ^{Cys}EYFP, lane 3: Peak I, lane 4: Peak II, lane 5: Peak IV, lane 6: Peak III.

NaCl concentration[19] and was restored after buffer exchange. In native PAGE (Fig. 4), the DNA moiety of the conjugate led to an increased electrophoretic mobility of the A24-^{Cys}EYFP conjugate in accordance with previous results from the synthesis of DNA- STV conjugates.[14] Determination of the molecular weight by SDS-PAGE revealed a single band with an apparent weight of *ca.* 36 kDa (Fig. 2, lane 4), which is in aggreement with the expected value. In contrast to unconjugated ^{Cys}EYFP, no dimer formation was observed for the A24-^{Cys}EYFP conjugate (Fig 2, lanes 3 and 4, respectively). The site specific conjugation of negatively charged DNA to the C-terminus might be responsible for the inhibition of dimerization due to sterical hindrance or electrostatic repulsion. In general, the synthesis of A24-^{Cys}EYFP led to isolated yields up to 50% with respect to the amount of A24 educt. The purity of the conjugates was greater than 95% as determined by PAGE analysis. Small impurities of uncoupled ^{Cys}EYFP could further be reduced by repeated chromatography.

Fluorescence measurements of A24-^{Cys}EYFP as well as of ^{Cys}EYFP in solution revealed that the emission intensity of the conjugate was higher then that of unconjugated protein (Fig. 6). Although the exact reasons of this observation are unclear at present, one may speculate that, for instance, the close proximity of DNA moiety alters the micro-environment and thus the optical properties of the EYFP's chromophore. Else the increase in fluorescence might be due to the decreased formation of EYFP dimers, and hence reduced quenching. Further elucidation of this phenomenon is under way.

The ability of the A24-^{Cys}EYFP conjugate to specifically hybridize with complementary ssDNA was investigated by

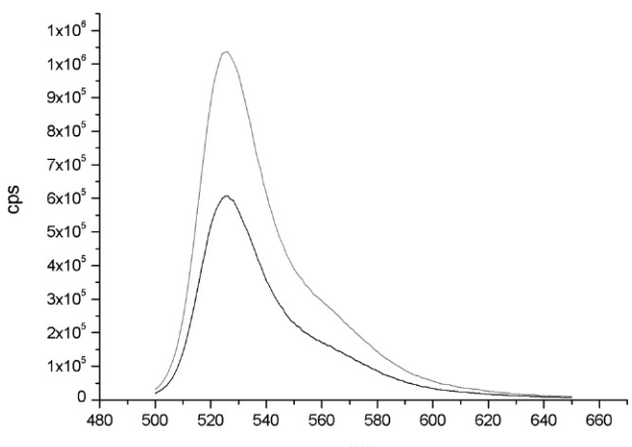

Fig. 6 Fluorescence of A24-^{Cys}EYFP conjugate and ^{cys}EYFP. Shown are the emission spectra of A24-^{Cys}EYFP (grey curve) and ^{cys}EYFP reference (black curve). Samples were diluted to 100 nM in conjugation buffer and excited at a wavelength of 488 nm.

gel-shift experiments as well as by the immobilization of the conjugate to STV-coated microtiter plates, containing the complementary biotinylated DNA cA24 as a capture probe.[20] In solid-phase hybridization, fluorescence signals of immobilized A24-^{Cys}EYFP conjugates were clearly distinguishable from negative controls, either lacking the cA24 or containing the non-complentary sequence cB21 (Fig. 7). To study the hybridization properties in homogeneous solution, the A24-^{Cys}EYFP conjugate was allowed to hybridize with a DNA-STV conjugate containing the complementary sequence cA24, covalently linked to the STV. As expected, the resulting supramolecular dimeric complex showed a reduced electrophoretic mobility due to the increase in molecular weight (Fig. 4, lane 7), as compared to the individual conjugates (lanes 6 and 8 in Fig. 4). Small amounts of other species are also observable in the gel. Beside some unhybridized cA24-STV and A24-^{Cys}EYFP conjugates, weak bands with even lower mobility are visible. Since similar bands are not visible in the control, containing A24-^{Cys}EYFP mixed with the non-complementary A24-STV conjugate (lane 9), the high molecular weight species are possibly due to non-specific binding of the A24-^{Cys}EYFP-cA24-STV dimer with other conjugates.

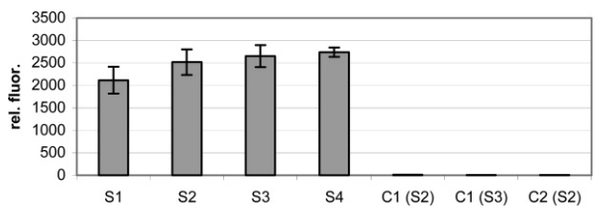

Fig. 7 Solid phase hybridization of A24-^{Cys}EYFP. Shown are the fluorescence signals of four different batches (S1–4) of A24-^{Cys}EYFP conjugates immobilized in STV-coated microplates, either functionalized with biotinylated complementary capture-oligomers cA24 (S1–4) or with a non-complementary sequence cB21 (C1). (C2) represents an additional control which lacks any capture oligonucleotide. The conjugate samples that were used in the negative controls are indicated in brackets.

Conclusion

We here described the synthesis of a novel species of semisynthetic DNA–protein conjugates using genetic engineering and organic coupling chemistry. A 24mer amino-modified oligonucleotide was coupled to a recombinant mutant of the enhanced yellow fluorescent protein. The resulting DNA–^{Cys}EYFP conjugate was purified to homogeneity, and was shown to maintain the physico-chemical properties of both components, *i.e.* the fluorescence of the ^{Cys}EYFP as well as the specific recognition properties of the DNA strand. We anticipate that such fluorescent DNA–protein conjugates can be used in the self-assembling, bottom-up fabrication of nanometer-scaled optically active molecular assemblies to be utilized as artificial light-harvesting complexes and antenna systems.

Experimental

Cloning and expression

The EYFP gene was amplified by PCR from the pEYFP vector (Clontech) using two primers (forward: 5′-CAGGATCCGTGAGCAAGGGCGAGGAGCTG-3′, reverse: 5′-GACGCAAGCTTCCTTAACAACCACCCTTGTACAGCTCGTCCATGCC-3), which add *BamH*I and *Hind*III restriction sites as well as codons for two glycine residues preceding the cysteine residue to the C-terminus of the gene (underlined) to the EYFP gene. PCR product and expression vector pRSET-A (Invitrogen) were digested with *BamH*I and *Hind*III (MBI Fermentas), purified with a spin column kit (Quiagen) and ligated with T4 DNA-ligase (New England Biolabs) for 1 h at room temperature. The ligation mixture was used to transform chemically competent *E. coli* BL21 cells which were then plated out on agar plates with 100 μg mL^{-1} Ampicillin for selection and incubated overnight at 37 °C. Single colonies were picked and grown to saturation overnight in 5 mL of LB-medium containing 100 μg mL^{-1} Ampicillin. Overnight cultures were used to innoculate 500 mL of the same media and protein expression

was induced by 0.5 mM IPTG when the cells were grown to an OD_{600} of 0.3. Cells were cultured at 25 °C overnight and harvested by centrifugation. The yellow pellet was resuspended in 20 mL of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 15 mM mercaptoethanol, 1 mM phenylmethylsulfonylfluoride, pH 8.0) followed by sonication. Cell debris were separated by centrifugation and the lysate was incubated with 5 mL of Ni–NTA-agarose (Quiagen) for 1 h on ice. The Ni–NTA-agarose was then transferred to an empty column and washed with 3 column volumes of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole, pH 8.0). Following, ^{Cys}EYFP was eluted with a total of 12 mL elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM Imidazole, pH 8.0) and the combined eluate was dialyzed against 500 mL of conjugation buffer (16.7 mM KH_2PO_4, 83.3 mM K_2HPO_4, 150 mM NaCl, pH 7.3) overnight with two buffer changes. Protein concentration was determined according to the Bradford method.[21] Glycerol was added to a final concentration of 30%, and the protein was aliquoted and stored at −80 °C at a typical concentration of 66 μM.

Conjugate synthesis and purification

To activate the DNA, 100 μL (500 μM) aminomodified oligonucleotide aA24 (5′-TCCTGTGTGAAATTGTTATCCGCT-3′, Thermo Electron) in H_2O was incubated 1 h at 35 °C with 100 μL conjugation buffer and 2 mg sSMCC, previously dissolved in 60 μL of DMF. 100 μL of a 1 M stock solution of DTT were added to 1 mL ^{Cys}EYFP (66 μM) and incubated for 30 min at 35 °C to reduce any disulfide bonds formed upon storage. Protein was concentrated by ultrafiltration using a 30,000 molecular cut-off filtration unit (Centricon 30, Millipore) to approx. 500 μL. Both the DNA and protein reaction mixtures were purified from low molecular compounds by two consecutive gel-filtration chromatography steps using commercially available columns (NAP5 and NAP10, Pharmacia) and conjugation buffer.

The purified DNA and protein solutions, each of which had a volume of 1.5 mL, were combined and incubated in the dark for at least 2 h. Following, the biomolecules were concentrated to approx. 200 μL and the buffer was exchanged to Elution buffer (20 mM Tris-HCl, pH 8.3) by ultrafiltration using a 30,000 Da molecular cut-off filtration unit (Centricon 30, Millipore). The conjugates were purified by anion-exchange chromatography on a MonoQ HR5/5 column (Pharmacia) by gradually increasing the NaCl concentration from 0 to 0.7 M. Peak fractions were pooled, concentrated and the buffer was exchanged to storage buffer (36.4 mM KH_2PO_4, 63.6 mM K_2HPO_4, 150 mM NaCl, 5 mM EDTA, pH 6.8). Conjugate absorbance at 260 and 280 nm was measured and quantified using calibration samples containing ^{Cys}EYFP and aA24 in a 1 : 1 molar ratio.

Conjugate characterization

Native and denaturing PAGE were performed on 10% acrylamide gels. SDS-PAGE was carried out according to the method described by Laemmli.[22] Gel-shift experiments were carried out with 10% acrylamide gels at 4 °C at a constant voltage of 150 V to prevent DNA denaturation due to excessive heating. For hybridization, 15 pmol of DNA-STV-conjugate and EYFP-DNA conjugate were incubated in a total volume of 6 μL storage buffer 1 h at room temperature. Gels were stained with SybrGold (Molecular Probes) and a silver developing kit (Bio-Rad), according to the manufacturer's instructions, and imaged with a UV-Vis gel documentation system (AlphaImager, Biozym) using appropriate filters.

DNA-directed-immobilization (DDI) was carried out as previously described[20] and fluorescence signals were detected with a microplate reader (Synergy HT, Bio-Tek) using a 488 nm excitation filter and a 525 nm detection filter.

Fluorescence emission spectra were carried out with a FluoroMax 3 instrument (Jobin Yvon) at an excitation wavelenght of 488 nm. Samples were previously diluted to 100 nM in conjugation buffer.

Acknowledgements

Financial support for this project was obtained by the Deutsche Forschungsgemeinschaft (DFG) through grant Ni-399/6-1.

References

1 N. Aratani, H. S. Cho, T. K. Ahn, S. Cho, D. Kim, H. Sumi and A. Osuka, *J. Am. Chem. Soc.*, 2003, **125**, 9668–9681.
2 T. S. Balaban, R. Goddard, M. Linke-Schaetzel and J. M. Lehn, *J. Am. Chem. Soc.*, 2003, **125**, 4233–4239.
3 R. Takahashi and Y. Kobuke, *J. Am. Chem. Soc.*, 2003, **125**, 2372–2373.
4 L. Hammarstrom, *Curr. Opin. Chem. Biol.*, 2003, **7**, 666–673.
5 M. Sykora, K. A. Maxwell, J. M. DeSimone and T. J. Meyer, *Proc. Natl. Acad. Sci. USA*, 2000, **97**, 7687–7691.
6 J. Hofkens, M. Cotlet, T. Vosch, P. Tinnefeld, K. D. Weston, C. Ego, A. Grimsdale, K. Mullen, D. Beljonne, J. L. Bredas, S. Jordens, G. Schweitzer, M. Sauer and F. De Schryver, *Proc. Natl. Acad. Sci. USA*, 2003, **100**, 13146–13151.
7 S. Jordens, G. De Belder, M. Lor, G. Schweitzer, M. Van der Auweraer, T. Weil, E. Reuther, K. Mullen and F. C. De Schryver, *Photochem. Photobiol. Sci.*, 2003, **2**, 177–186.
8 J. Qu, N. G. Pschirer, D. Liu, A. Stefan, F. C. De Schryver and K. Mullen, *Chem.–Eur. J.*, 2004, **10**, 528–537.
9 T. Weil, E. Reuther, C. Beer and K. Mullen, *Chem.–Eur. J.*, 2004, **10**, 1398–1414.
10 C. M. Niemeyer and C. A. Mirkin (Eds.), *NanoBiotechnology: Concepts, Methods and Applications*, VCH, Weinheim, 2004.
11 C. M. Niemeyer, *Science*, 2002, **297**, 62–63.
12 C. M. Niemeyer, *Trends Biotechnol.*, 2002, **20**, 395–401.
13 C. M. Niemeyer, J. Koehler and C. Wuerdemann, *ChemBioChem*, 2002, **3**, 242–245.
14 C. M. Niemeyer, T. Sano, C. L. Smith and C. R. Cantor, *Nucleic Acids Res.*, 1994, **22**, 5530–5539.
15 M. Ormo, A. B. Cubitt, K. Kallio, L. A. Gross, R. Y. Tsien and S. J. Remington, *Science*, 1996, **273**, 1392–1395.
16 R. Heim, *Methods Enzymol.*, 1999, **302**, 408–423.
17 G. T. Hermanson, *Bioconjugate Techniques*, Academic Press, 1996.
18 S. Inouye and F. I. Tsuji, *FEBS Lett.*, 1994, **351**, 211–214.
19 R. M. Wachter and S. J. Remington, *Curr. Biol.*, 1999, **9**, R628–629.
20 C. M. Niemeyer, L. Boldt, B. Ceyhan and D. Blohm, *Anal. Biochem.*, 1999, **268**, 54–63.
21 M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248–254.
22 U. K. Laemmli, *Nature*, 1970, **227**, 680–685.