Site-specific labeling of DNA–protein conjugates by means of expressed protein ligation†

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Site-specific bioconjugation of protein thioesters with a DNA oligonucleotide was achieved by Expressed Protein Ligation (EPL) and the new thiol group formed upon EPL in the conjugate was selectively coupled with small molecule labels using maleimide chemistry.

Protein microarray technology holds great promise for a variety of applications in biomedical diagnostics and proteomics, particularly where the multiplex analysis of various parameters from very small amounts of sample volumes is desired.[1] Because of the intrinsic instability of many proteins, which often leads to denaturation upon spotting, chemically mild procedures are required to allow the efficient immobilization of the proteins at chemically activated surfaces. To address this problem, we have developed the DNA-directed immobilization (DDI) of semisynthetic DNA–protein conjugates, using stable DNA microarrays as the immobilization matrix to which the DNA–protein conjugates bind by means of Watson–Crick base pairing (for a schematic representation of DDI, see Fig. 3A).[2]

Although DDI is a versatile and highly specific immobilization method, the chemical coupling of the proteins of interest with the DNA-tag is often cumbersome because the stoichiometry and regioselectivity of coupling are difficult to control.[2a,3] To circumvent this problem, we[4] and others[5] have recently developed the expressed protein ligation (EPL)[6] of intein-fusion proteins (**1** in Fig. 1) with nucleic acid oligomers bearing an N-terminal cysteine moiety **2**. We reasoned that the newly formed mercaptomethyl group at the ligation site of **3**, originating from the S–N acyl-shift during EPL,[6] might be a suitable target for further chemical modification of the DNA–protein conjugate by means of maleimide coupling (Fig. 1). This approach, which has so far only been applied in the ligation and labeling of small peptides,[7] would enable the incorporation of additional functional groups of interest (*i.e.*, labels for spectroscopic read-out) at the predetermined protein site without compromising the conjugate's structural and functional integrity.

To investigate the feasibility of our concept, we tested whether thiol-reactive dye **4** (Fig. 1), which has previously been used for labeling biomolecules for Surface Enhanced Resonance Raman Scattering (SERRS) analysis,[8] would, indeed, bind to DNA–protein conjugate **3** subsequent to its synthesis by EPL. To this end, **3** was generated by EPL from thioester-containing maltose-binding protein (MBP) **1** and the cysteine-modified DNA oligomer **2** (5′-cysteine-CCT GTG TGA AAT TG-3′), by a procedure similar to that previously described.[4c] Purified **3** was then incubated with an excess of **4** and the reaction mixture was analysed using anion-exchange chromatography. As shown in Fig. 2A, only a single peak III revealed the characteristic absorption at both 280 nm (protein and DNA) and 386 nm (dye **4**), thus indicating the successful coupling of **4** with conjugate **3** to yield the desired SERRS dye–DNA–MBP conjugate **6**.

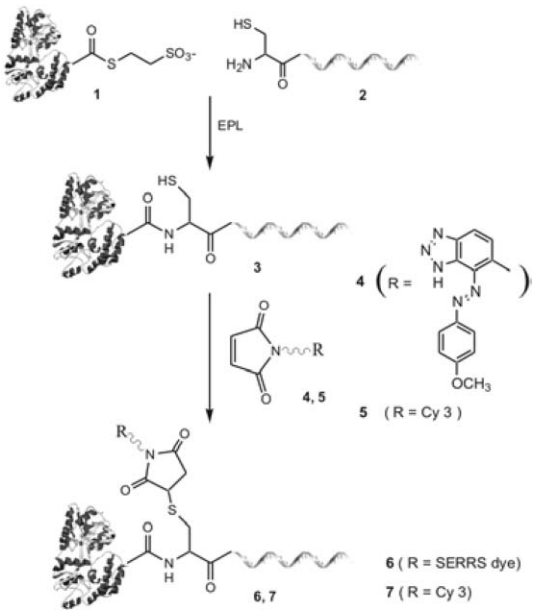

Fig. 1 The synthesis of DNA–protein conjugate **3** by EPL leads to the formation of a new thiol group at the ligation site which is subsequently addressed by thiol-reactive maleimide derivatives of the SERRS dye **4** or the fluorescent Cy3 dye **5**.

The high sensitivity of SERRS is attributed to the huge enhancement of Raman scattering when suitable chromophores are adsorbed onto the roughened metal surfaces of, usually, silver or gold,[9] and maleimide dye **4** was specifically designed as a label for biomolecule detection by SERRS.[10] To further prove the integrity and functionality of MBP–DNA conjugate **6**, it was allowed to bind to microtiter plates through specific DNA-hybridization,[11] and detection of bound conjugates was achieved by SERRS. As shown in Fig. 2B, spectrum (a), the SERRS spectrum obtained upon 514 nm laser excitation revealed the characteristic fingerprint signals of the chromophore **4** within **6**. No such SERRS signals were observable in control experiments, in which **6** was incubated in wells containing non-complementary

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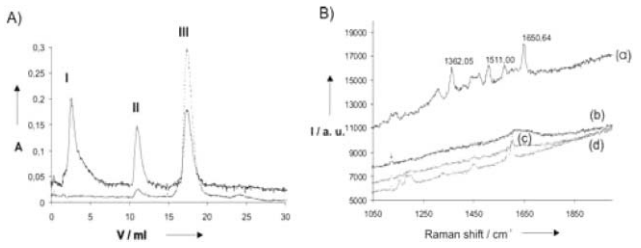

Fig. 2 A) FPLC chromatogram of the MBP–DNA conjugate **6**, obtained from **3** by maleimide coupling with SERRS dye **4**. Black and gray dotted lines indicate the absorbance measured at 386 and 280 nm, respectively. Peak III represents conjugate **6**. Note that peaks I, II represent unreacted dye **4**. B) SERRS spectra of the conjugate **6** taken at 514.5 nm after immobilisation in microplate wells containing (a) complementary, (b) non complementary, and (c) no capture oligonucleotides. Spectrum (d) represents a control in which unlabeled conjugate **3** was allowed to bind to complementary capture oligonucleotides.

(spectrum b), or no capture (spectrum c) oligonucleotides. Additional controls in which **3** was allowed to bind to complementary capture oligomers also yielded no SERRS signals (spectrum d in Fig. 2B). These results therefore confirm that **6** retains its binding capabilities to complementary DNA, and, because the MBP used here did not contain any other cysteine residues, the results also prove that dye **4** was indeed coupled to the newly formed thiol moiety generated in the course of the EPL-based synthesis of MPB–DNA conjugate **3**.

The applicability of the site-specific labeling was further investigated by coupling of the thiol-reactive fluorescent dye Cy3 (**5** in Fig. 1). Incubation of **3** with **5** yielded the desired conjugate **7** (Fig. S1a)[11] as confirmed through fluorescence analysis of purified **7** (Fig. S1b). The biological activity and the applicability of **7** as a reagent for diagnostics were investigated in a DDI-based model sandwich immunoassay (Fig. 3A). To this end, DNA microarrays were prepared on glass, as previously described,[2e,12] which contained two capture oligonucleotides, **8** and **9**, complementary and non-complementary, respectively, to the DNA in **7**. Various concentrations (1–100 nM) of conjugate **7** were then allowed to bind to the DNA array (step I, in Fig. 3), and subsequently, the immobilized conjugates were used for binding of the model analyte, *i.e.*, mouse anti-MBP **10**, also applied in variable concentrations, ranging from 1–100 nM (step II). Detection of captured analyte was carried out using an antibody against mouse IgG, labeled with Cy5–streptavidin conjugate (**11** in step III). Finally, the fluorescent signals of the microarrays were measured using both the Cy3- (λ_{Exc} = 550 nm, λ_{Em} = 570 nm) and the Cy5-specific channels (λ_{Exc} = 649 nm, λ_{Em} = 670 nm) of a microarray scanner.

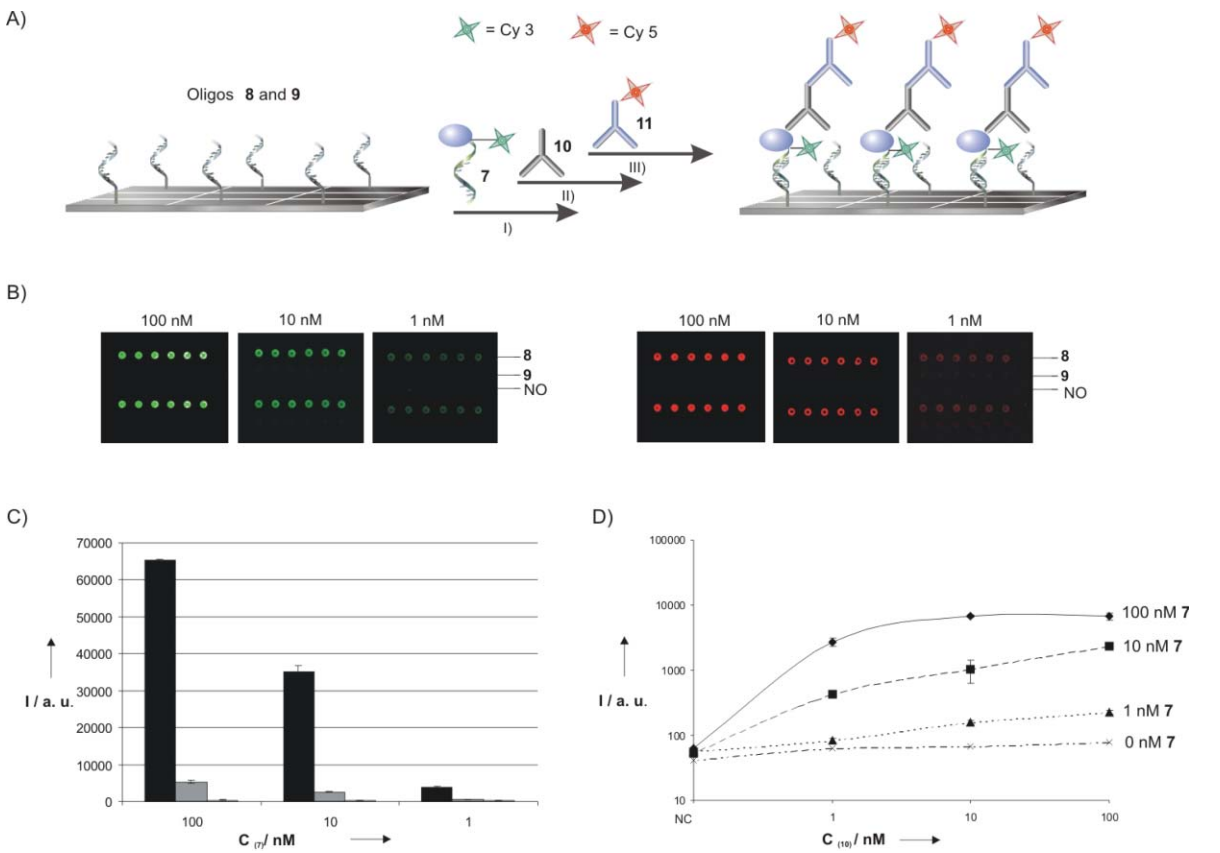

Fig. 3 A) Schematic drawing of DNA-directed immobilization of Cy3–MBP–DNA conjugate **7** and its use as a capture reagent in the microarray-based sandwich immunoassay. B) Cy3- (left) and Cy5- (right) fluorescence signals obtained from the immunoassay after steps I–III; note that the two images show the same chip. C) Quantification of Cy3 fluorescent signals of **7**, measured at spots containing complementary (black bars), non-complementary (gray) or no capture (white bars) oligonucleotides. Various concentrations of **7** were used during immobilization. D) Quantification of detection conjugate **11** (Cy5 fluorescent signals) in dependency of both the concentration of analyte **10** (*x*-axis) and **7** (curves).

The fluorescence signals observed in this two-color immunoassay (Fig. 3B) clearly indicated the biological functionality of the DNA as well as the intact folding of the MBP moiety of **7**.[13] Quantification of the Cy3 signals of **7** (left image in Fig. 3B, and bars in Fig. 3C) revealed only weak signals of the control spots (containing non-complementary capture oligomer **9**), confirming that the immobilisation of **7** predominantly occurred due to specific Watson–Crick base pairing. It was also evident that even the lowest concentration of **7** (1 nM, corresponding to $\sim$50 ng ml^{-1}) was clearly detectable (Fig. 3C). The average interassay standard deviation calculated from the Cy3 signal intensities obtained from four different slides was only about 6%, thus validating earlier reports on the high reproducibility of the DDI method.[2b]

The applicability of the two-color DDI-immunoassay for the detection of analyte substances was tested by applying various concentrations of the analyte, anti-MBP antibody **10**, to a set of arrays comprising immobilized **7**. The results shown in Fig. 3D revealed that detection of the lowest analyte concentration (1 nM) was even possible at the spots generated by DDI of 1 nM of **7**. Moreover, the shape of dose-response curves obtained for 10 nM and 100 nM of **7** suggested that even lower than 1 nM concentrations of analyte can be detected.

Thus, these results clearly demonstrate that the site-specific conjugation of DNA and protein-thioesters by EPL, followed by the site-specific labeling of the newly generated thiol group using maleimide-containing small-molecule tags, allows one to generate oligofunctional biomolecular probes. These can be used as reagents in DDI-based immunoassays, thereby enbling, for instance, the direct quantification of both capture probe and analyte. Because our labeling method enables the quantitative, two-color detection of antigens, we anticipate that the strategy described here should be applicable to the screening of libraries of recombinant proteins for applications in biomedical diagnostics, functional proteomics and other fields of chemical biology[14] and nanobiotechnology.[2d]

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