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Synthesis of protein–nucleic acid conjugates by expressed protein ligation

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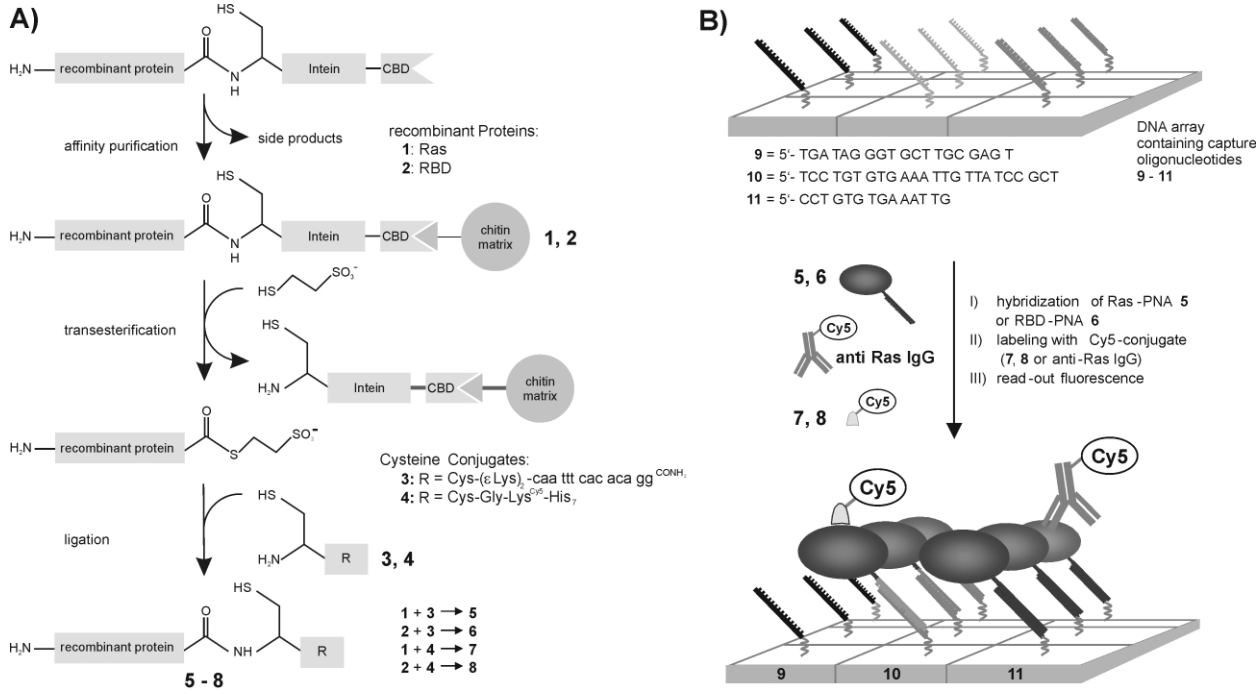
The synthesis of covalent conjugates of proteins and polyamide nucleic acids (PNA) is accomplished by expressed protein ligation of intein-fusion proteins and a PNA-cysteine conjugate.

Protein microarrays are currently being explored as tools in proteome research and miniaturized multianalyte clinical diagnostics.[1] A general problem of protein biochips, however, concerns their preparation by stepwise, robotic immobilization of multiple proteins at chemically activated surfaces, which is often obstructed by the intrinsic instability of many proteins, and thus, loss of functionality. DNA-directed immobilization (DDI)[2] of proteins provides a chemically mild procedure for the highly parallel attachment of multiple proteins to a solid support using DNA microarrays as immobilization matrices (Scheme 1). The high efficiency of adsorption, the reversibility and site-selectivity of DDI enables a variety of applications, including the fabrication and reconfiguration of biosensor surfaces and the production of microarrays containing both nucleic acids and proteins for genome and proteome research.[2c] Recently, DNA-directed immobilization strategies, for instance, employed synthetic pyranosyl-RNA oligomers as recognition elements for the addressable immobilization of antibodies and peptides,[3] and PNA-hapten conjugates in functional genomics for identifying members of a small molecule split-pool library which bind to protein targets.[4]

To further expand the scope of DDI-related methods, however, efficient methodologies are required to allow for chemically mild and regioselective coupling of nucleic acids with proteins. We report here on the expressed protein ligation of intein-fusion proteins with a conjugate comprising a polyamide nucleic acid (PNA) coupled with a short peptide sequence containing an N-terminal cysteine (Scheme 1A). Previous work has shown that peptide–nucleic acid conjugates are available.[5,6] However, the conjugation of small peptide–PNA constructs to proteins by expressed protein ligation has not been described before.

Expressed protein ligation has previously been used for the synthesis of a variety of proteins.[7,8] In a first step (Scheme 1A), the target protein fused to the construct of an intein and a chitin binding domain (CBD) is expressed in *E. coli*. This latter domain allows the affinity purification of the intein-fusion protein using a chitin matrix. Liberation from the column is achieved by mercaptoethansulfonic acid (MESNA), which produces a C-terminal thioester of the target protein. This protein can be ligated to a peptide containing an N-terminal cysteine. Here, two different conjugates, cysteine–PNA conjugate (**3**) and Cy5-labelled peptide (**4**), were fused either to the



Scheme 1 Schematic drawing of the generation of protein-conjugates **5–8** by expressed protein ligation (A) and the DNA-directed immobilization (DDI) of PNA conjugates **5** and **6** on a model DNA microarray (B).

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Ras protein (leading to conjugates **5** and **7**, respectively) or the Ras binding domain of c-Raf-1 (RBD) (leading to **6**, **8**).

PNA **3** was synthesized on solid-phase according to the Boc-strategy[9] by using Boc/Z-protected PNA building blocks and Fmoc-Lys(Boc) and Boc-Cys(Trt). Standard acidolysis liberated the PNA–cysteine conjugate **3**, which was subsequently purified by RP-HPLC. Peptide **4** was synthesized by solid phase peptide synthesis as previously described[10] and was purified by HPLC to homogeneity. The fluorophore Cy5 was coupled to the peptide via the Lys side chain (Becker et al., submitted).

As indicated in Scheme 1, **3** and **4** were ligated to Ras (**1**) and RBD (**2**) resulting in conjugates **5** (generated from **1** + **3**), **6** (**2** + **3**), **7** (**1** + **4**), and **8** (**2** + **4**). Chemical ligation was carried out as described earlier.[10a] In brief, peptides (1mM) and proteins (1 mM) activated with 250 mM MESNA and 3% ethanethiol were mixed in physiological buffer (100 mM NaCl; 50 mM Tris, pH 7.5; 5 mM $MgCl_2$; 2.5 mM DTE) and incubated over night at room temperature. The constructs were purified by dialysis and analyzed using SDS gel electrophoresis and mass spectrometry. Ligation efficiency was estimated to be up to 60%, however, it should be noted that the ligation reaction and the purification was not optimized at this stage because the chip analysis described below allowed an unequivocal detection of the correct conjugates. RBD was renatured by incubating for a short period in the above physiological buffer. Purified Ras had to be renatured in the presence of GDP.[10a] For the generation of activated Ras (Ras·GTP) the non-hydrolizable GTP analogue GppNHp was used. The exchange of GDP by GppNHp was carried out as previously described.[11]

Ras is a small G-protein which plays a central role in cellular signal transduction. In its GTP bound form Ras triggers downstream components such as c-Raf-1 (RBD), while Ras·GDP does not bind to RBD. Therefore, RBD is a perfect monitor for the detection of Ras·GTP even in the presence of Ras·GDP.

To prove the functionality of conjugates **5,6** in DDI-based applications, we studied their solid-phase hybridization capabilities using a model DNA microarray. For this, glass slides were modified with a dendritic intermediate layer as previously described,[12] and 5′-amino modified oligonucleotides **9–11** (Scheme 1B) were covalently coupled to the solid support. Note that sequences **10,11** are fully complementary to the PNA, but differ in length, while sequence **9** is not complementary, and thus functions as a negative control for specific hybridization of conjugate **5,6**. As indicated from the signal intensities shown in Fig. 1, conjugates **5,6** specifically hybridized with the complementary capture oligomers, immobilized on the chip. The intensity of the Cy5-IgG labelled sample is stronger, probably because two Cy5 dyes are coupled to the monoclonal antibody. Moreover, DNA chip analysis clearly allowed the differentiation between Ras in its activated and its non-active form (reactions C2 and C3 in Fig. 1).

In conclusion, we have shown that the rapid and efficient semi-synthesis of conjugates of proteins and nucleic acids can be achieved by expressed protein ligation. Due to the convenient synthesis of PNA-Cys conjugates, as opposed to DNA-Cys conjugates, we chose PNA as a model system. The chemical ligation leads to nucleic acid–protein conjugates which are well-defined with respect to stoichiometric composition and regiospecific linkage. This method has several advantages over conventional chemical coupling techniques, and thus, constitutes a major improvement for further developments of artificial multi protein arrangements as well as nanostructured hybrid assemblies.[2c,d] Since DNA–peptide conjugates are also available,[5] it is now possible to produce, rapidly and automatically, PNA– as well as DNA–protein conjugates from libraries of recombinant proteins. Thus, the chemical ligation described here will be useful for a wide variety of applications, ranging from proteome research and clinical diagnostics to the arising field of nanobiotechnology.[2c]

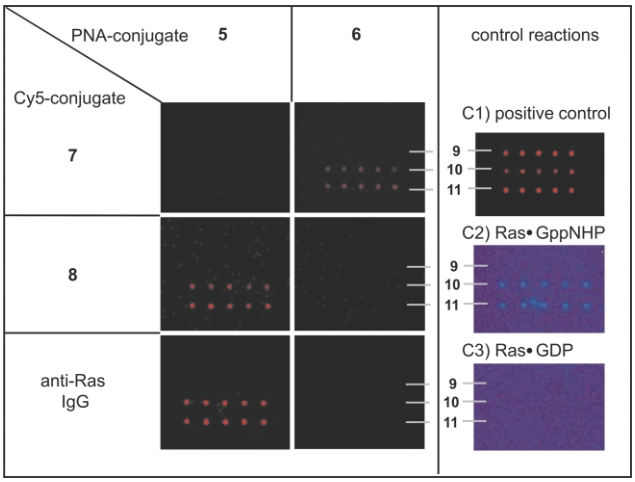

Fig. 1 Solid-phase hybridization experiments with conjugates **5–8**. Each sub-array is comprised of three rows of different DNA oligomers, **9–11**. Sequence **9** is not complementary, and thus, represents a negative control for the specific hybridization of PNA conjugates **5** and **6**, while sequences **10** and **11** are fully complementary to the PNA, but differ in length. In the outer right column three control experiments are shown: Two Cy5-labelled oligonucleotides **12** (Cy5- TGA GCG TTC GTG GGA TAG T) and **13** (Cy5- AGC GGA TAA CAA TTT CAC ACA GGA), fully complementary to chip-immobilized **9** and **10,11**, respectively, were hybridized to the chip as a positive control (image C1); chip-immobilized RBD-PNA conjugate **6** was probed with unlabelled Ras in either its activated (Ras·GppNHP, image C2) or its inactive form (Ras·GDP, image C3), and subsequently, the sandwich complex was detected with the Cy5-labelled anti-Ras IgG.

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