Preparation of Symmetrical and Unsymmetrical DNA – Protein Conjugates with DNA as a Molecular Scaffold

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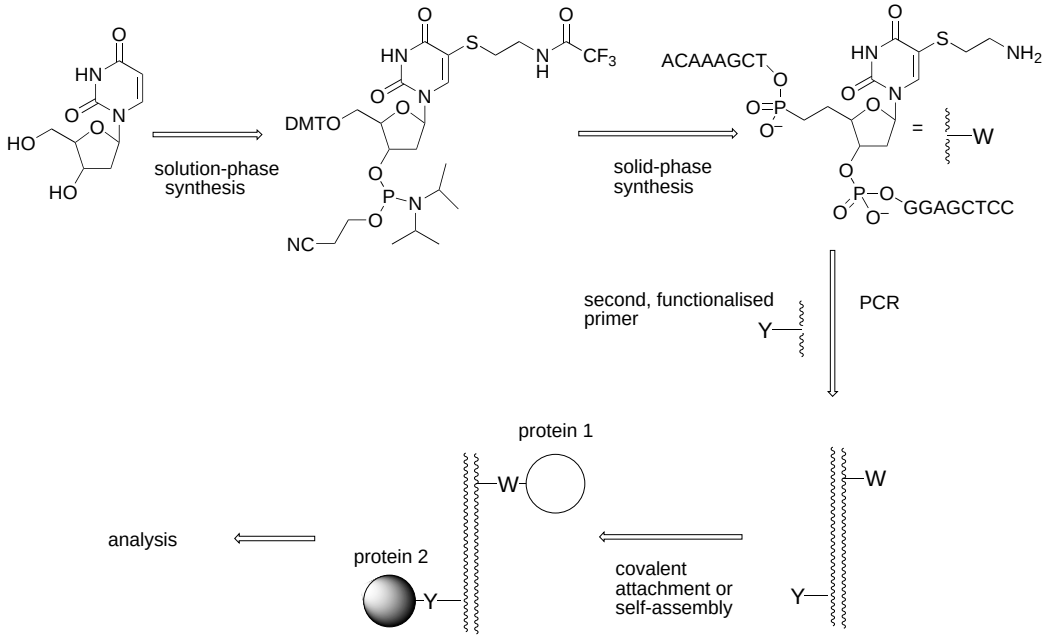
As the functions of individual components of the cell are more and more elucidated (e.g. by genomics), the challenge of understanding the factors involved in macromolecular organisation become more pressing. The first generation of model systems to study protein organisation are being developed, most notably in the work of Niemeyer and others, with DNA as a molecular scaffold[1] onto which proteins are attached, either covalently[2] or noncovalently.[3] In the latter studies, polymerase chain reaction (PCR) with 5′-biotin-derivatised oligonucleotides was used to give double-stranded (ds) DNA products. When streptavidin (STV) was added to these modified dsDNAs, a mixture of oligomeric complexes rapidly formed through self-assembly. Here we describe a variation on this approach by using internally modified PCR primers that results in the formation of DNA – protein dumbbells, and the extension of the approach to the preparation of the first unsymmetrical system with two different proteins attached to the DNA scaffold.

The approach used to prepare DNA – protein constructs (Scheme 1) is a variation on the PCR method employed by Niemeyer et al.[3a] The primary difference is that the linker used to attach the protein is at an internal base position in the oligonucleotide primers instead of at the 5′ end. This was intended to give a more rigid and defined point of attachment. Consequently, PCR primers were prepared that incorporated 2′-deoxyuridines modified with a C-5 side chain, which act as mimics of the natural base 2′-deoxythymidine (dT) and do not significantly diminish the potential for complementary Watson – Crick base pairing. The side chain modification allows the introduction of a thiol, a carboxylate, a primary amino, or a biotin group into the DNA oligomer.



Scheme 1. *Strategy for the organisation of proteins into a defined relative spatial orientation with DNA as a molecular scaffold. DMT = 4,4′-dimethoxytrityl.*

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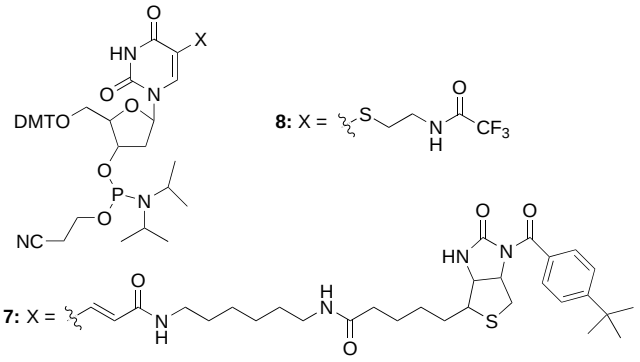
Six oligonucleotides, **1** – **6** (Table 1), were synthesised by solid-phase phosphoramidite chemistry,[4] and characterised by negative-ion electrospray mass spectrometry (data not shown). The biotinylated residue **7** (dTB) was incorporated at the central position of each of three oligonucleotides **1**, **3** and **5**, while the amino-modified residue **8**[5] (dTA) was incorporated at the central position of oligonucleotide **6**. A series of PCRs using these primers gave dsDNA 203, 292 or 318 base pairs long, when

*Table 1. DNA sequences of the synthesised oligonucleotides **1** – **6**.*

Compd	Sequence	Length	Modification
1	CAATTTCCAT**X**CGCCATTCAG	21	X = dTB
2	CAATTTCCATTCGCCATTCAG	21	none
3	GTACCCAA**X**TCGCCCTA	17	X = dTB
4	GTACCCAATTCGCCCTA	17	none
5	CGACGGCCAG**X**GAGCGCGCGT	21	X = dTB
6	ACAAAGCT**X**GGAGCTCC	17	X = dTA



plasmid pBluescript II SK(−) or a derivative, pEX (see Experimental Section), was used as a template. With the oligonucleotides **2** and **4** and pEX as a template, an unmodified 203-bp control PCR product was formed. By using oligonucleotide **2** with modified oligonucleotide **3**, the same 203-bp PCR product was formed, but now with a biotinylated base incorporated at one end. With two modified primers (**1** and **3**) the 203-bp dsDNA was synthesised with biotinylated residues incorporated at both ends. A longer (292 bp) doubly biotinylated PCR product was also prepared from pEX by using primers **1** and **5**. A bifunctionally modified 318-bp PCR product, incorporating an amino-modified base at one end and a biotinylated base at the other, was prepared from the template pBluescript II SK(−) using primers **1** and **6**.

In initial experiments, the assembly of DNA – protein conjugates between biotinylated oligonucleotides and STV was investigated. In each experiment an approximate tenfold molar excess of STV over the biotinylated DNA was used, and the products were analysed by agarose gel electrophoresis (Figure 1). As previously reported by Niemeyer et al.,[3] the addition of STV to the biotinylated DNA samples led to the formation of new DNA bands in the higher molecular weight region of the agarose gel, indicating the formation of DNA – STV conjugates. However, none of the distinct fingerprint-like patterns, which were postulated to be due to distinct distributions of oligomeric species,[3] were observed. Furthermore, from the intensity of the DNA bands on the gel, the major proportion of the DNA appeared to be present in only one form.

To study the structures of the doubly biotinylated DNA – STV conjugates, samples were imaged by using contact mode atomic force microscopy (AFM) (Figure 2). Dilute aqueous solutions of the conjugates were air-dried on mica and then covered with *n*-butanol prior to imaging. The *n*-butanol reduces capillary forces which affect imaging in air, and also may dehydrate the sample,

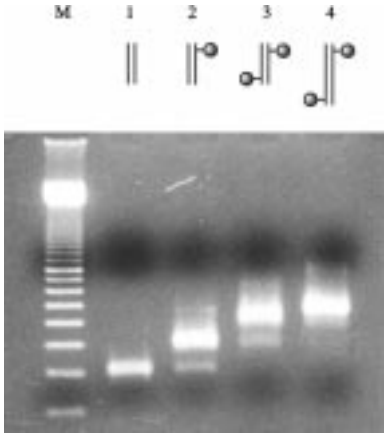


Figure 1. *DNA – STV conjugates on a 1.5 % agarose gel after electrophoresis and staining with 0.3 g mL^{-1} ethidium bromide. The agarose gel was run in 1 × TAE buffer at 100 V and 80 mA. DNA-containing fragments were visualised on a short-wave transilluminator. M: 100-bp DNA molecular weight marker (Pharmacia); lane 1: 203-bp unmodified PCR product and STV; lane 2: 203-bp singly biotinylated PCR product and STV; lane 3: 203-bp doubly biotinylated PCR product and STV; lane 4: 292-bp doubly biotinylated PCR product and STV.*

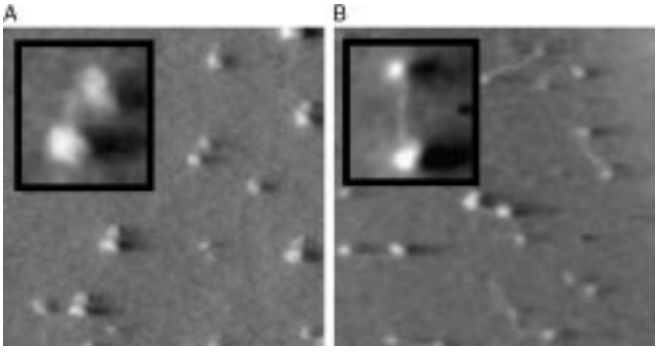


Figure 2. *Atomic force microscopy (AFM) images of the DNA – STV dumbbells. Representative images are shown. A: 203-bp DNA – STV dumbbell (scan area: 6700 × 6700 Å; inset: 900 × 950 Å). B: 292-bp DNA – STV dumbbell (scan area: 5000 × 5000 Å; inset: 1200 × 1350 Å). AFM images were obtained in the contact mode under* n*-butanol.*

making it more rigid. Figure 2 A shows multiple copies of a construct formed by having monovalent STV attached to each end of the dsDNA (referred to as dumbbells), and unattached molecules of STV (used in excess). The intervening DNA in the dumbbell is partially observed. It is clear from the expanded image (Figure 2 A, inset) that the DNA is bent. Indeed, the separation between the centres of the two proteins is 490 Å compared to the predicted separation of 690 Å.[6]

In order to confirm the interpretation of Figure 2 A, a similar sample of the longer (292 bp) DNA – STV conjugate was prepared (Figure 2 B). Again, multiple copies of DNA – STV dumbbells can be seen. The expanded image (Figure 2 B inset) shows a selected single dumbbell chosen because the DNA scaffold is straight. Indeed, the measured distance between the centres of the STVs (960 Å) is close to the predicted value of 990 Å. However, in Figure 2 B several dumbbells are seen which show significant bending of the scaffold DNA. This may be due to the natural flexibility of DNA, but could also be due, in part, to

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the contact mode of imaging used to obtain these AFM images dragging the samples across the mica surface during the image acquisition.

Niemeyer et al. reported observing similar DNA – STV dumbbell structures,[3a] but in mixtures which included longer conjugates (where an STV effectively links two pieces of biotinylated DNA into linear or circular structures) and branched divalent and trivalent species. The amounts of the different species were shown to vary as a function of the molar ratio of STV to DNA, with dumbbells most evident when the ratio was high (5:1). The apparent presence of only dumbbells reported above may be due to the ratio of STV to DNA used ($>$ 10:1). It may also be a consequence of the linker being attached internally in the primer and so making the STV less accessible to biotin on another strand of DNA. Further experimentation is required to fully explore this idea.

In order to study many aspects of enzyme association, it is desirable to form DNA – protein assemblies incorporating two different proteins. This is a logical extension of the subject but has not previously been demonstrated. In order to make such an unsymmetrical DNA – protein dumbbell, primers incorporating differently functionalised linkers were used in the PCR. By using the biotinylated and amino-modified primers **1** and **6**, the bifunctionally modified 318-bp PCR product was generated and then activated by cleavage of the group protecting the amino function. The free amino-modified oligonucleotide was functionalised with the heterobifunctional cross-linker *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) and coupled to freshly activated papain.[7] This conjugate was then associated with an approximate tenfold molar excess of STV to give the desired DNA – STV – papain conjugate, which was analysed by AFM. Figure 3 shows an image obtained of one such unsymmetrical

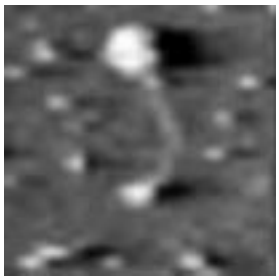

Figure 3. *AFM image of an unsymmetrical 318-bp DNA – STV – papain dumbbell (scan area: 1300 × 1300 Å). AFM image obtained in the contact mode under* n-*butanol.*

dumbbell. The larger STV molecule ($M_r \approx 60$ kDa) at one end of the DNA scaffold can clearly be differentiated from the smaller papain molecule ($M_r \approx 23$ kDa) at the other end. As was also seen in previous images, there is some bending of the scaffold DNA, with a separation between the two proteins of 1000 Å compared to the predicted value of 1080 Å.

The preparation of DNA – protein dumbbells by using internally modified PCR primers, and the organisation of two different proteins on a DNA scaffold are novel contributions to the exciting and developing area of protein organisation on DNA scaffolds. It is clear from the AFM images that even over a few hundred base pairs, DNA cannot be simplistically considered a rigid rod, giving a fixed separation of proteins. This, however, does not detract from the use of DNA as a scaffold to organise very closely spaced proteins at distances similar to those expected in multienzyme complexes. Consequently, these results open up the way for further studies, such as on the effect of enzyme organisation on substrate channelling.

Experimental Section

Oligonucleotide synthesis: Oligonucleotides used in this study were prepared by Oswel DNA Services (Southampton, UK). The sequences of the unmodified, biotinylated and amino-modified oligonucleotides **1** – **6** are shown in Table 1. Biotinylated oligonucleotides **1**, **3** and **5** were obtained by incorporation of the commercially available phosphoramidite **7** at the appropriate point in the synthesis. The amino-modified oligonucleotide **6** was prepared by the central incorporation of the phosphoramidite **8**. During the final stage of oligonucleotide synthesis, the protection of the amino group was cleaved to give the free amino-modified oligonucleotide.

Polymerase chain reaction: The primers for the PCR were chosen to anneal to regions of either pBluescript II SK(−) or pEX—a derivative of pBluescript II SK(−) lacking 26 bp in the multiple-cloning region. Plasmid pEX was prepared by digestion of pBluescript II SK(−) with *Eco* RI and *Xba* I, filling the recessed 3′ ends by using the Klenow fragment of DNA polymerase I, then ligating the blunt ends by using T4 DNA ligase. PCRs were carried out on a Hybaid OmniGene Thermal Cycler with 0.5 mL Treff microcentrifuge tubes. Reaction mixtures (50 μL) were set up including each primer (5 μL, 10 μM), 10 × reaction buffer (5 μL), template DNA (5 μL, 10 ng), dNTPs (10 mM of each), and sterile water (28 μL). *Taq* DNA polymerase (1 μL, 1 U) was added to these components and then a covering layer of sterile mineral oil (30 μL). The PCR block was run on the following program: 1 cycle: 94 °C, 4 min; 35 cycles: {94 °C, 1 min; 50 °C, 1 min; 72 °C, 1 min}; 1 cycle: 72 °C, 5 min. The mineral oil was removed from the samples by repeated pipetting of the reaction mixture onto parafilm. The crude PCR mixtures were purified by spin-column chromatography using a 1-mL column packed with Sephadex G-25, before being concentrated in vacuo and redissolved in 0.1 M HEPES buffer, pH 8.2 (30 μL).

Preparation of DNA – protein conjugates: Biotinylated DNA was conjugated to STV by incubation of the DNA (8 μL, ca. 0.5 μg) with STV (5 μg, ca. tenfold excess) in 0.1 M sodium carbonate buffer (12 μL), pH 9.5, at room temperature for 30 min. The free amino-modified DNA (20 μL, 40 μg) was incubated with a solution of SPDP (0.2 mg) in acetonitrile (20 μL) under a covering layer of mineral oil (35 μL) at 50 °C for 16 h. The mineral oil was removed and the reaction mixture concentrated in vacuo. This was then redissolved in 0.1 M sodium carbonate buffer (50 μL), pH 9.5, before being purified by spin-column chromatography as described above. Freshly activated papain (60 μL, 0.15 mM) and SPDP-derivatized oligonucleotide (20 μL) in 0.1 M sodium carbonate buffer (20 μL), pH 9.5, were incubated at room temperature for 16 h. The conjugates were analysed by agarose gel electrophoresis and AFM.

Atomic force microscopy: AFM was carried out on an East Coast Scientific (Cambridge, UK) atomic force microscope, using silicon nitride cantilevers (Digital Instruments) with a spring constant of 0.38 N m^{-1} and square-pyramidal tips with a radius of curvature of

50 nm. Samples of the respective DNA – protein conjugates were desalted by spin-column chromatography using 1-mL Sephadex G-25 columns, before being serially diluted to an appropriate concentration for imaging individual molecules (ca. 1 – 5 nM). A 2-μL drop of each sample was placed onto a freshly cleaved mica surface and then dried in a vacuum desiccator for 2 h. *n*-Butanol (HPLC grade, Aldrich) was then added to cover the sample, prior to topographic and error signal images being obtained in the contact mode.

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