

Efficient preparation of internally modified single-molecule constructs using nicking enzymes

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ABSTRACT

Investigations of enzymes involved in DNA metabolism have strongly benefited from the establishment of single molecule techniques. These experiments frequently require elaborate DNA substrates, which carry chemical labels or nucleic acid tertiary structures. Preparing such constructs often represents a technical challenge: long modified DNA molecules are usually produced via multi-step processes, involving low efficiency intermolecular ligations of several fragments. Here, we show how long stretches of DNA (>50bp) can be modified using nicking enzymes to produce complex DNA constructs. Multiple different chemical and structural modifications can be placed internally along DNA, in a specific and precise manner. Furthermore, the nicks created can be resealed efficiently yielding intact molecules, whose mechanical properties are preserved. Additionally, the same strategy is applied to obtain long single-strand overhangs subsequently used for efficient ligation of ss- to dsDNA molecules. This technique offers promise for a wide range of applications, in particular single-molecule experiments, where frequently multiple internal DNA modifications are required.

INTRODUCTION

Single-molecule experiments on DNA have grown in popularity after providing unprecedented insights into polymer physics (1), molecular motors (2) and other DNA-interacting proteins (3). Recent technical advances have promoted the development of setups which combine techniques in order to allow the simultaneous acquisition of independent parameters (4–6). For example optical tweezers have been used to apply forces in Förster resonance energy transfer (FRET) measurements (7). The

more sophisticated experiments require in turn increasingly complex DNA substrates often with multiple modifications. In order to elucidate the mechanisms by which DNA is replicated, transcribed or repaired such modifications can be of chemical and structural nature. While for the preparation of short modified DNA constructs, the annealing of complementary oligonucleotides will suffice (7,8), this method is not suitable for preparing long labeled substrates, such as plasmids (9) or constructs used in magnetic and optical tweezers (10) experiments. Therefore, other strategies based on the enzymatic incorporation of chemically modified bases are in use, such as random inclusion via PCR (11,12) or 5'- and 3'-end labeling (13). Nevertheless, intermolecular ligation of multiple fragments is by far the most frequently applied method to assemble long internally labeled constructs (9,10,14). However, the efficiency of the ligation drops drastically when increasing the number of individual fragments. This makes it technically difficult to create long constructs carrying multiple labels at distant loci in sufficient quantity. Moreover, the number of non-nicked molecules is very low which is a considerable disadvantage in cases where intact DNA is required, e.g. experiments involving supercoiled DNA (9,15).

Alternatives to internally label nucleic acids that yield a higher proportion of intact molecules are peptide nucleic acids (PNAs) (16–18) and DNA triplex probes (19). Such probes offer a high degree of flexibility for the positioning of the internal label. Disadvantageous, however, is the non-covalent and thus to some extent unstable nature of the attachment, in particular at applied mechanical load. Furthermore, the probe locally changes the DNA structure, which in turn alters the mechanical properties, and can act as a barrier for DNA translocating motor enzymes (20). In order to achieve internal covalent and site-specific labeling, nicking enzymes have been employed. One way to introduce single or multiple labeled bases at a specifically generated nick is to use nick translation, which has been successfully employed in single-molecule DNA barcoding (21–23).

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Table 1. Oligonucleotides used for cloning, internal labeling and hairpin construction

Name	Sequence
top-cloning	tcgagc <u>ctca</u> gctcatgtca <u>tcctcagcac</u> acttgaccct cagctcagct agcctcagcc tacaatcacc tcagcg
bottom-cloning	aattc <u>gctga</u> ggtgattgta <u>ggctgaggct</u> agctgagctg agggccaagt gt <u>gctgagga</u> tgacatgagc <u>tgagggc</u>
biotinx6	tcagc <u>T</u> catg tcatcc <u>T</u> cag cacact <u>T</u> gac cctcagc <u>T</u> ca gctagcc <u>T</u> ca gctacaa <u>T</u> c acc
biotinx2	tcagctcatg tcatcctcag cacact <u>T</u> gac cctcagc <u>T</u> ca gctagcctca gctacaa <u>T</u> c acc
BstXI-Fill	tcagctcatg tcatcctcag cacact <u>T</u> gac cctcagc <u>T</u> ca <u>gccgagggac gactaCCAGA TGCCTGGgag</u> <u>ttgccgattt tcggcaactc CCAGGCATCT GGtagtcgtc cctcgtagcc</u> tcagcctaca atcacc
hairpin-partI	[Btn]tttttgggag cactacgttc ggactagtgt actctgactt gagact <u>ttttg</u> tctcaagtc
hairpin-partII4	agagtacact agtccgaacg tagtgctccc 44(t) <u>atgc</u>
hairpin-partIII0	agagtacact agtccgaacg tagtgctccc 44(t) <u>atgcatgccc</u>

Capital and underlined 'T's represent biotin-modified thymines and a 5' biotinylated phosphate [Btn]. For the oligomers top-cloning and bottom-cloning, the 5 BbvCI recognition sites are shown underlined. For the BstXI-Fill oligomer, underlined bases mark the hairpin used to create the Y-junction molecule and capital letters mark the BstXI recognition site. For the hairpin-partI oligomer, underlined bases mark the hairpin end-loop consisting of four thymidines. For the hairpin-partII4 and hairpin-partIII0 oligomers, the 4 and 10 bp long ends used for the ligation are underlined, respectively.

Alternatively nicking enzymes can also be used to excise a short stretch of single strand DNA out of long duplex DNA (24). The created gap can subsequently be refilled using a chemically modified DNA oligomer. Although this procedure involves a complex purification procedure, it nevertheless set the trend for a range of studies based on proximal nicking sites. It offers a higher degree of control during the reaction and an increased flexibility in choice and position of the desired modification (25,26).

We here simplify and develop further the latter strategy: we demonstrate how long stretches of DNA (>50 bp) can be modified to produce complex constructs for single-molecule experiments. Multiple different chemical modifications can be precisely located internally along DNA, even at distant sites. The nicks created can be resealed efficiently yielding intact molecules, which support supercoiling in magnetic tweezers experiments. Furthermore, our strategy allows to conveniently introduce structural features, demonstrated here by creating a branched DNA molecule. We achieve efficient ligation of ss- to dsDNA fragments using long (10 nt) overhangs, produced with the aid of nicking enzymes (27,28), to incorporate a hairpin into a DNA construct.

MATERIALS AND METHODS

DNA and enzymes

Oligonucleotides were designed with the aid of the software mfold (29) to exclude sequences that would lead to the formation of strong secondary structures. All oligonucleotides (Table 1) were purchased from Sigma-Aldrich (St Louis, MO, USA). Enzymes were from New England BioLabs (Ipswich, MA, USA) and used as suggested by the manufacturer unless otherwise stated. To prepare the plasmid pNLrep, which carries a region with five equally spaced (15–16 bp) BbvCI sites, the oligos top-cloning and bottom-cloning (Table 1) were hybridized and ligated between the EcoRI and XhoI sites of the linearized recipient vector, a 6868-bp long derivative of pBluescript II SK+ (Stratagene, La Jolla, CA, USA).

Internal labeling and biochemical tests

To prepare DNA with internal modifications, pNLrep was nicked using Nt.BbvCI for 1 h. When required, the plasmid was linearized in the same reaction. Subsequently 1/10 volume of 200 mM Tris-HCl, pH 8.0, 400 mM ethylenediaminetetraacetic acid (EDTA) and 100 M excess of modified oligo, were added to the mixture. The sample was heated to 80°C for 2 min and cooled to 20°C at a rate of 1°C/min to allow the oligomer carrying the modification to anneal into the gap created.

Successful internal biotinylation was tested using pulldown and band-shift assays. For the band-shift assays 11 fmol of biotinylated DNA was incubated with 150 fmol streptavidin in phosphate buffered saline (PBS) for 20 min at room temperature. Alternatively, the same amount of modified DNA was incubated with 50 fmol of streptavidin conjugated quantum dots 625 (Q-dots) (Invitrogen) in PBS for 10 min. For the pulldown assay, 1.5-fold more of the DNA sample was used to compensate for unspecific binding of DNA to the beads: 16.5 fmol of biotinylated DNA were incubated for 10 min with 0.032 fmol prewashed streptavidin-coated magnetic beads (M-280, Invitrogen, Carlsbad, CA, USA) in PBS containing 0.1 mg/ml BSA and 100 mM NaCl. After pelleting the beads within a magnetic rack, the supernatant was collected. All DNA samples were analyzed on 1% agarose gels in Tris-Acetate-EDTA (TAE) buffer or 5% polyacrylamide in Tris-Borate-EDTA (TBE) buffer and subsequently stained with ethidium bromide.

Substrates for magnetic tweezers experiments were prepared as previously described (12). Fluorescein- or digoxigenin-modified attachment handles were prepared by PCR in the presence of fluorescein- or digoxigenin-modified dUTPs (Roche, Basel, Switzerland). Subsequent digestion produced ≈600 bp fragments with 5'-overhangs on one of the ends. These modified fragments were ligated to a 9-kb long internally modified fragment, which was digested during the nicking reaction to produce compatible overhangs.

AFM, magnetic tweezers and fluorescence measurements

AFM imaging was performed in intermittent contact mode in air using a JPK Nanowizard atomic force

microscope (AFM) and Olympus cantilevers (AC160TS). In all, 3 μ l of DNA sample diluted to a concentration of 0.5 ng/ μ l in buffer (10 mM Tris-HCl, pH 8, and 5 mM MgCl₂) was placed onto freshly cleaved mica supports for 5 min, washed twice with buffer and dried under a gentle stream of nitrogen. For AFM imaging of Q-dot-DNA assemblies, Q-dots were dialyzed against 10 mM Tris-HCl, pH 8, for 30 min prior to incubation with DNA (see above). AFM images were analyzed using WSxM 3 software (30). Magnetic tweezers experiments were carried out as previously described (31). Briefly, the digoxigenin-modified end of the DNA construct was bound to the anti-digoxigenin antibodies-coated glass surface of a flow cell. At the fluorescein-modified DNA end, an anti-fluorescein antibody-coated magnetic bead was bound. A pair of external magnets was used to apply force onto the bead and to stretch the DNA. The bead position was determined from video images using 3D particle tracking (31). For fluorescence measurements, the setup was additionally equipped with a Sapphire 488-50 laser (Coherent Inc., Santa Clara, CA, USA) and a DU897-COO-BV EMCCD camera (Andor Technology plc., Belfast, UK).

Hairpin constructs involving ligation of ss- to dsDNA

A total of 40 bp hairpins were prepared by annealing the oligomers hairpin-partI with hairpin-partII4 (4 nt 3'-overhang) or hairpin-partIII10 (10 nt 3'-overhang) and subsequent ligation (Table 1). The 3'-ends of the hairpins were complementary to 4 or 10 nt 3'-overhangs of dsDNA handle fragments. Handles were produced by PCR by incorporating either a BstXI or a BbvCI site into one of the PCR primers (Table 1) followed by digestion with either BstXI or Nt.BbvCI (Figure 6a). Hairpins were mixed at 10-fold molar excess over the handles and ligated for 1 h at room temperature or 16°C overnight ligation. Prior to ligation, the sample with the 10 bases long 3'-overhangs was additionally heated to 50°C for 5 min and directly put on ice to displace the small 10 nt fragment from the overhang. The final ligation products were analyzed and the gel was purified using 2% agarose gels. Handles for magnetic tweezers experiments with a digoxigenin-modified tail were prepared as described above.

RESULTS

Sequence design and modification

Our aim was to develop a method for internal labeling of long DNA substrates which would fulfil the following requirements: (i) the internal modification should be accomplished with almost 100% efficiency and only require a single additional purification step (regular PCR product clean-up), since substrates for single-molecule experiments require additional modifications and procedural steps, each of which substantially reduces the yield. (ii) The method should allow to incorporate multiple modifications at defined positions. For example, the so-called rotor-bead assay (10), that allows direct DNA twist measurements, requires a rigid attachment of large particles,

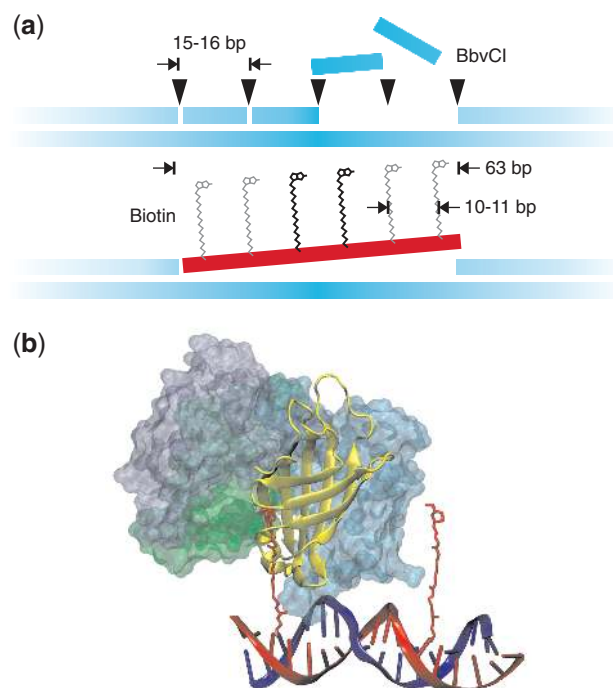


Figure 1. Schematic representation of the internal labeling method. (a) A DNA sequence which incorporates five equally spaced BbvCI recognition sites (black triangles) is nicked only at one of the two strands using either the nicking enzyme Nt.BbvCI or Nb.BbvCI. This results in the formation of short 15–16 bases long fragments. Denaturation and subsequent hybridization, in the presence of a DNA strand (shown in red) that is complementary to the resulting 63 bp gap and that carries the desired internal modifications (e.g. two or six biotins as depicted), lead to an efficient replacement of the original fragments with the labeled fragment. The spacing between the internal modifications of 10–11 bp ensures that they extrude in the same direction from the DNA. (b) Model of a streptavidin tetramer bound to an internally biotinylated DNA molecule (Streptavidin PDB id: 1MK5; the bound monomer is illustrated as a yellow ribbon while for the other subunits the surface representation was used. DNA PDB id: 2BNA). The attachment of the streptavidin tetramer to only one of the biotins was arbitrarily chosen.

such as Q-dots or fluorescent beads, which can be best achieved via multiple biotin–streptavidin linkages.

We based our method on a previous elegant strategy (24), where nicking enzymes were used to nick the DNA at two proximal sites which results in the formation of a short (22 bp) gap. The gap was subsequently refilled with DNA oligomers carrying the desired modification. This attempt, however, required two purification steps—one upon gap formation and one upon refilling. In addition, it was restricted to relatively short replacement sections.

In order to achieve both a single-step procedure and the replacement of longer DNA stretches, we thought of using multiple, i.e. more than two nicking enzyme sites placed at a short distance from each other (Figure 1a). The introduction of multiple nicks should allow to create long gaps. On the other hand, it should also increase the efficiency of the replacement reaction since the long insert carrying the modifications should be thermodynamically more stable than the multiple short fragments generated after nicking.

Based on these considerations we designed a DNA sequence with five binding sites of BbvCI in direct

repeat, which were 15–16 bp apart (Figure 1 and Table 1). Nicks at the BbvCI sites can be introduced using the enzymes Nt.BbvCI and Nb.BbvCI, which nick either the top or the bottom strand, respectively, of the recognition sequence. Using these enzymes, one can freely choose the specific strand to be modified but also modify both strands in a sequential manner. We ensured the absence of strong secondary structures in the replace sequence (see ‘Materials and Methods’ section) in order to facilitate the annealing of the modified oligomer. The designed sequence was inserted into a host vector to yield the plasmid pNLrep (see ‘Materials and Methods section’).

To internally modify pNLrep it was nicked using Nt.BbvCI, followed by addition of an excess of the oligomer with the internal modification that would replace the original 15–16 bp fragments. The sample was heated to 80°C, subsequently slowly cooled down to 20°C in order to efficiently displace the short fragments and ensure a proper and efficient hybridization of the oligomers with the modifications. For the latter, we used oligomers carrying 2 or 6 biotin-labeled thymidines, which were 10–11 bp spaced (Figure 1b and Table 1). This ensured that on average all biotins extrude from the DNA into the same direction, which should improve and facilitate the attachment of larger objects such as Q-dots and fluorescent beads through multiple bonds.

Labeling and religation efficiency

To confirm the internal biotinylation, we carried out an electrophoretic mobility retardation assay. pNLrep was nicked using Nt.BbvCI and simultaneously cut into three linear fragments of which one (0.8 kbp in length) carried the repetitive BbvCI sites. Nicking of the 0.8 kbp fragment led to a small mobility retardation compared with the unnicked fragment (Figure 2a, Lane 2). A subsequent spin column purification caused a much larger retardation specific to the 0.8 kbp fragment due to release of the short 15 bp fragments causing a gap to be formed (Figure 2a, Lane 3). If, however, a replace reaction in the presence of an oligomer with internal modification was carried out prior to purification, the labeled 0.8 kbp fragment runs at the position of the nicked fragment (Figure 2a, Lane 4), demonstrating the successful incorporation of the oligomer. To additionally validate the internal labeling, streptavidin was added to the labeled DNA, causing the 0.8 kbp fragment specifically to disappear most likely due to an increased mobility causing an overlap with the 5 kbp fragment. Furthermore, we carried out a pulldown experiment with streptavidin-coated magnetic beads, which again sequestered the modified fragment but not the unmodified fragments demonstrating the specificity of the labeling reaction. All experiments were performed with both replace oligomers (carrying two and six biotins) and provided equal results. Both test experiments revealed a high efficiency of the single step reaction with the labeled DNA being the dominating reaction product.

In addition to the labeling efficiency we also tested the religation efficiency, i.e. to what extent the nicks in the DNA template can be resealed after the replace reaction.

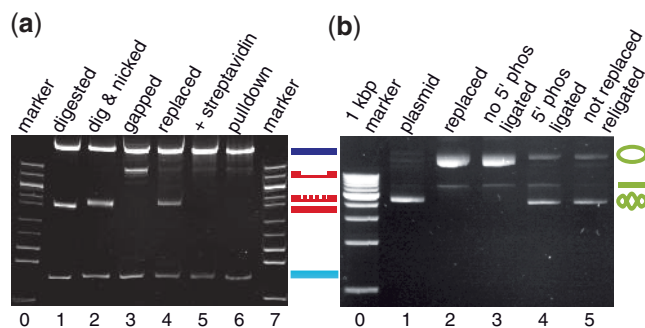


Figure 2. Internal modification and religation efficiencies. (a) Polyacrylamide gel electrophoresis of DNA samples in the course of the internal labeling procedure. (Lane 1) pNLrep after digestion with MluI and AatII yielding a 0.8-kb fragment (red line) that carries the region to be replaced as well as a 0.4-kb (light blue line) and a 5-kbp fragment (dark blue line). (Lane 2) pNLrep after simultaneous digestion with MluI, AatII and Nt.BbvCI. The nicking of the 0.8-kb fragment (represented by the fragmented red line) can be seen as a slight mobility decrease. (Lane 3) Sample from lane 2 after column purification, which leads to gap formation within the 0.8-kb fragment causing a large mobility alteration (gapped red line). (Lane 4) Sample from lane 2 after the replacement reaction with oligo biotinx2, during which the 0.8-kb fragment becomes internally biotinylated, and subsequent column purification. The inserted oligo is stably bound and therefore displays the same mobility as the nicked fragment in lane 2. (Lane 5) Sample from lane 4 with >10-fold molar excess of streptavidin added. (Lane 6) Pulldown assay with sample from lane 4 (see ‘Materials and Methods’ section). (Lanes 0, 7) 100 bp step DNA ladder, starting at 400 bp with an additional 517 bp band. (b) (Lane 1) pNLrep. (Lane 2) pNLrep after nicking and internal biotinylation with oligo biotinx2. (Lane 3) Sample from lane 2 after ligation. (Lane 4) pNLrep after internal biotinylation with 5'-phosphorylated biotinx2 oligo and religation. (Lane 5) pNLrep after nicking with Nt.BbvCI and religation. Positions of supercoiled, nicked and linearized plasmid species are indicated by corresponding symbols at the right side. (Lane 0) 1 kb step DNA ladder with the shortest fragment starting at 1 kb.

This is particularly important for experiments where intact substrates are necessary, e.g. when involving supercoiled DNA (9,10,15). The religation efficiency was tested by ligating the circular, internally modified plasmid and separating the reaction products on an agarose gel in the presence of ethidium bromide (Figure 2b). The religation efficiency was found to be strictly dependent on the 5'-phosphorylation of the insert oligomer. For a non-phosphorylated insert, no detectable religation could be observed which demonstrates that the gap was efficiently filled with the modified oligomer and not with the short fragments left over from nicking. For a phosphorylated insert, most (>90%) of the originally nicked plasmid became unnicked upon ligation (compare only the bands for the nicked species due to different staining of nicked and unnicked DNA with ethidium bromide). The amount of unnicked plasmid was found to be comparable to a control, where the plasmid was simply nicked and directly religated.

Single-molecule experiments

Beyond the biochemical tests we also verified the labeling and religation efficiency directly in single-molecule experiments. Substrates for these investigations afford more elaborate preparations and the practical use of the

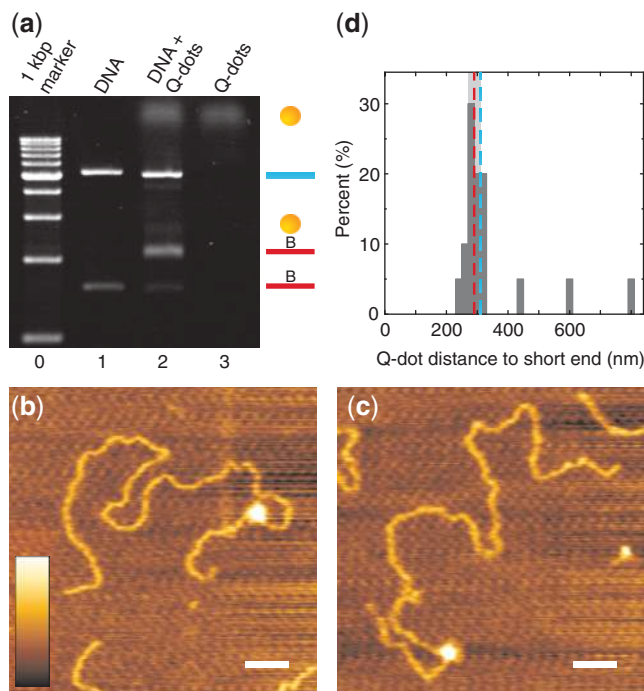


Figure 3. Site-specific attachment of Q-dots to internally biotinylated DNA. (a) Band-shift assay of Q-dot binding to DNA. (Lane 0) 1 kb step DNA ladder with the shortest fragment starting at 1 kb. (Lane 1) pNLrep after digestion with BamHI, PspOMI and Nt.BbvCI and internal biotinylation with oligomer biotin_x2 (Figure 2a). (Lane 2) Sample from Lane 1 with 5-fold molar excess of streptavidin coated Q-dots added. (Lane 3) Sample containing Q-dots only. Symbols on the right side indicate Q-dots (yellow spheres), the short biotinylated fragment (red line) and the long non-biotinylated fragment (blue line). (b and c) AFM images of Q-dots bound to DNA. The colour scale corresponds to a height-range of 1 nm, and the scale bar corresponds to 100 nm. (d) Histogram of the Q-dot position measured from the nearest DNA end. The expected Q-dot position at 920 bp (310 nm) (blue dashed line) is within the double confidence interval (light grey band) of the experimentally determined mean (290 ± 20 nm, red dashed line).

internal labeling method can only be judged in such measurements. In particular, we tested the ability to bind a fluorescent particle (Q-dot) specifically to the predetermined location within an internally biotinylated DNA molecule. This is a common task e.g. when assembling the above-mentioned rotor-bead assay (10). We first repeated the mobility retardation assay (Figure 3a) using this time Q-dots (instead of streptavidin). Q-dot binding exhibited similar efficiency with streptavidin binding with only very little uncomplexed DNA remaining. The shift in migration was specific only to the modified molecule and was more pronounced compared with streptavidin due to the larger size of the Q-dots.

We tested the correct localization of the Q-dots on DNA in AFM experiments. We prepared a modified fragment 5.8 kbp long with biotins being located 920 bp away (≈ 310 nm) from one end and incubated it with Q-dots. Their binding to DNA could be readily visualized in AFM images (Figure 3b and c). Image analysis provided that 85% of the Q-dots were found at the expected location along the DNA molecules: with a

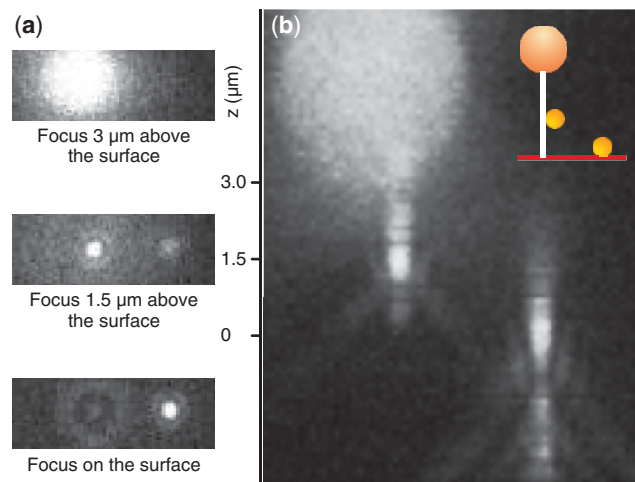


Figure 4. Magnetic tweezers experiment with internally attached Q-dot. DNA molecules with an attached Q-dot bound through internal biotinylation were stretched in the vertical direction within a magnetic tweezers setup (see Inset in Figure 4b). Q-dots were detected by fluorescence. (a) Fluorescence images taken at different focal (z -) positions above the surface of the flow cell. At 0, 1.5 and 3 μ m a surface-bound Q-dot, the internally attached Q-dot and the weakly auto-fluorescent magnetic bead were within the focal position of the objective. This was in agreement with the expected positions, since the Q-dot was internally attached at 4.4 kb (≈ 1.5 μ m) while the full length of the molecule was 9 kb (≈ 3.0 μ m). (b) z -stack of a linear section across the surface- and the DNA-bound Q-dot at different z -positions. (Inset) schematic representation of the experimental configuration. DNA was drawn as a white line, Q-dots as small yellow spheres and the magnetic bead as a large orange sphere.

mean of 290 ± 20 nm, the expected length is within the error of the measurement (Figure 3d) and its standard deviation is in agreement with previous contour length determinations of nucleic acids (32).

In addition, we tested Q-dot binding in a magnetic tweezers experiment that mimics a rotor-bead experiment (10). The DNA is attached at one end to a glass surface and at the other end to a magnetic bead. An externally applied field allows the stretching of the DNA away from the surface (31). To achieve the binding of a fluorescent particle at an internal site, one needs three different attachments. One of them is the internal biotinylation approximately in the center of the molecule to allow Q-dot binding. The two additional attachments are required for the ends, which were realized by ligating handles carrying digoxigenin- and fluorescein-modified bases to an internally biotinylated 9 kb DNA fragment, to allow binding to the antibody covered glass surface and the magnetic bead, respectively. The DNA was first bound to Q-dots then to magnetic beads and introduced into the flow cell of the magnetic tweezers. The presence of a Q-dot was verified by fluorescence microscopy (Figure 4a). Taking a z -stack of fluorescence images also the position of the Q-dot on the DNA could be determined. Free surface bound Q-dots served as a reference in these experiments (Figure 4b). In addition, we carried out DNA supercoiling measurements (15). Only intact, i.e. unnicked DNA molecules reduce in length as response to external twist. From a total of 20 DNA

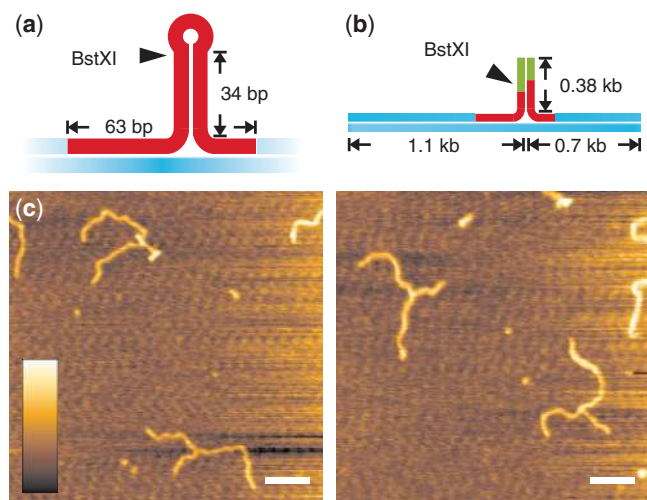


Figure 5. Inserting structural modifications. Schematic representation: (a) a hairpin (shown in red) containing a BstXI site (black triangle) was introduced via the single-step replace reaction. (b) After digestion with BstXI, a 500-bp DNA fragment (shown in green) was ligated to the junction arm. (c) AFM images of the resulting Y-shaped molecules; the lengths of the three branches (355 ± 5 , 250 ± 15 and 140 ± 14 nm) agree with the expected values (390, 240 and 130 nm) for the exception of a discrepancy of the longest branch. The colour scale corresponds to a height range of 1 nm, the scale bar corresponds to 100 nm.

molecules found in the magnetic tweezers, 70% carried an internally attached Q-dot at the expected position ($\sim 1.5 \mu\text{m}$ above the surface). In all, 50% of all molecules were intact and 35% of all molecules were both intact, i.e. supercoiled, and carried a Q-dot. We note that on average $\sim 50\%$ of standard magnetic tweezers substrates without internal modifications, which are produced by intermolecular ligations, are found to be intact. Thus, the internal modification scarcely affects the fraction of intact molecules.

Structural modifications

Beyond internal chemical labeling, our method allows also to introduce in a one step reaction internal tertiary structural modifications, such as hairpins or junctions, which are common substrates in single-molecule experiments (15,33). Inserting an oligomer (BstXI-Fillin), which contains a sequence that folds into a hairpin (Figure 5a) in a replace reaction yields a double-stranded branched molecule. The target sequence of the restriction enzyme BstXI was incorporated in the hairpin. This was used to extend the hairpin branch by digesting it and ligating a ≈ 400 bp long fragment to it, resulting in an asymmetric branched molecule (Figure 5b). The successful addition of the ≈ 400 bp long branch could be easily verified by agarose gel electrophoresis (data not shown) as well as by AFM imaging where Y-shaped molecules with the correct proportions were obtained (Figure 5c).

ssDNA to dsDNA ligation at nicking enzyme generated overhangs

For many substrates in single-molecule experiments, the ligation of single- to double-stranded DNA is required. This involves in particular experiments with

DNA hairpins to investigate nucleic acid folding (34) or enzymes that act at replication forks (35,36). The single-stranded tails of the hairpins are ligated to longer and more rigid dsDNA handles. The ligation efficiency strongly depends on the length of the sticky end of the handle and it is rather low for the typical 4nt overhangs generated by restriction enzymes. Longer overhangs can be generated using Autosticky PCR (37), which supports efficient ssDNA to dsDNA (38). However, this method is restricted to 5'-overhangs only and requires rather expensive primers.

A simpler alternative, which allows to generate both 3' and 5' overhangs, can be achieved by using nicking enzymes (27,28). Here, we incorporated a BbvCI site into the primer that is used to produce the dsDNA handle by PCR (Figure 6a). After nicking the handle using Nt.BbvCI followed by a short denaturation, a 10 nt long 3'-overhang is formed, which supports highly efficient attachment of a 40-bp hairpin at its 3'-end in a subsequent annealing/ligation reaction (Figure 6a). As a control, we used a second hairpin, which was ligated to only a 4-nt long 3'-TACG-overhang, generated by digesting an appropriate dsDNA handle using BstXI. In contrast to the construct with the 10 nt overhang, no detectable ligation product could be found in this reaction (Figure 6a). Both ligations for 1 h at room temperature and at 16°C overnight provided comparable results.

The correct covalent attachment of the hairpin to the dsDNA handle was also verified in a magnetic tweezers experiment. To support the tethering of the molecule, a 600 bp tail with digoxigenin-modified bases was ligated to the dsDNA handle and a biotin was added to the 5'-end of the hairpin. A sufficiently high force was applied on the hairpin construct, which allowed its transient unfolding and the folded and unfolded state to be approximately equally populated (Figure 6b). The difference in DNA extension between the two states was 38 nm corresponding to ≈ 80 nt as expected.

DISCUSSION

Here, we have presented a simple and efficient method based on nicking enzymes to generate chemical (Figure 1) and structural (Figure 5) modifications internally within long DNA molecules as well as long single-stranded overhangs capable of efficient ssDNA to dsDNA ligation (Figure 6). Additionally, we have shown that this method can be easily integrated into the normal workflow of preparing DNA substrates for single-molecule experiments, providing a high amount of intact substrates.

Site-specific internal labeling based on nicking enzymes is more efficient than intermolecular ligations of multiple fragments (39). In addition, the DNA structure is minimally distorted confined to the site of the modification. This is advantageous over methods that use an external binding domain for the modification as provided by PNAs and triplexes (40,41) or DNA-binding proteins (42,43).

Compared with previous internal labeling based on nicking enzymes (24), our method that relies on

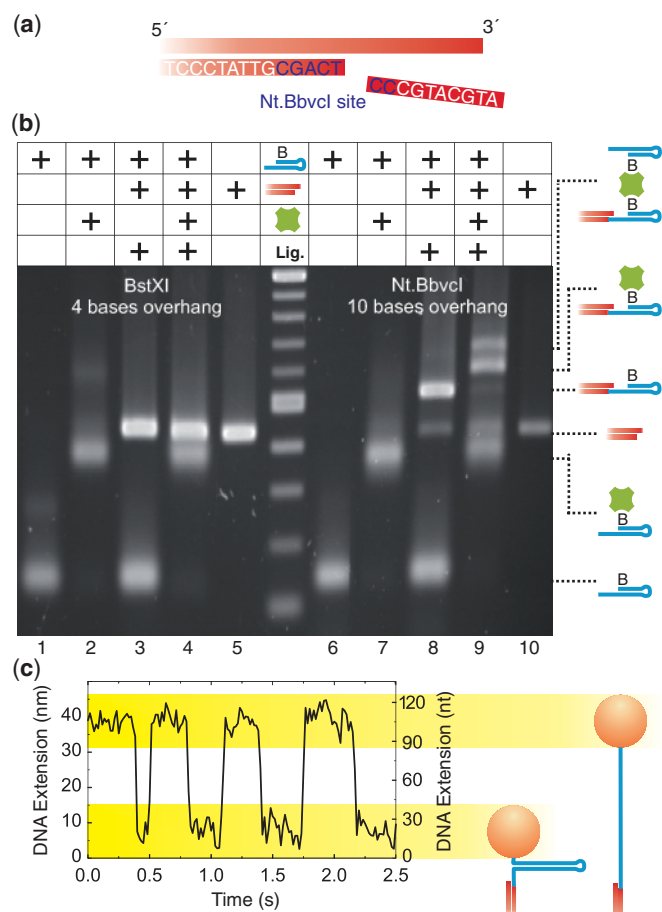


Figure 6. ssDNA to dsDNA ligation at nicking-enzyme-generated overhangs. (a) Schematic representation of overhang generation. A BbvCI recognition site (blue letters) was incorporated near the DNA end in such a way that nicking with Nt.BbvCI generates a 10-bp fragment at the 5'-end. (b) Agarose gel of DNA fragments, ligation products and streptavidin-induced band shifts. The biotinylated 40-bp hairpin, the 430-bp dsDNA handle and streptavidin are represented by a blue, red and green symbol, respectively. 'Lig.' indicates where a ligation for 1 h at room temperature was carried out. Positions of the reaction products are marked at the right side. (Lanes 1–5) reaction products for the 4nt overhang generated by BstXI. (Lanes 6–10) reaction products for the 10nt overhang generated by Nt.BbvCI. The lane in the middle is a 100-bp size marker ladder with the shortest fragment starting at 100-bp and 100-bp size difference between all subsequent fragments (and an additional band at 517bp). The success of the ssDNA to dsDNA ligation was confirmed by the streptavidin-induced band-shift, in which the desired product specifically shifted only in the case where a 10nt 3'-overhang had been used. (c) Magnetic tweezers experiment with the generated hairpin construct. The molecule was held at the critical force where the closed and the opened states of the hairpin (as illustrated by the sketches) were nearly equally populated. The change in height between the two states was ≈ 38 nm as expected for a 40-nt hairpin.

multiple nicking sites requires only a single purification step and is capable of modifying rather long stretches of DNA. Furthermore, there are also several advantages compared with nick translation which is very successful at incorporating a single-modified base close to a specifically introduced nick (21) or to label a stretch of DNA of somewhat uncontrolled length downstream of a nick with multiple fluorescent bases (44). Our method goes beyond that: it allows to place single or multiple labels

over a well-defined DNA stretch of ~ 60 bp in length, at any desired locations with nearly no limitations. It also allows to introduce structural modifications and the labeling is very specific and restricted to that particular gap to which the oligomer is targeted.

Despite the amount of nicks introduced in the substrate DNA, a high amount of intact molecules is obtained after ligation (Figure 2b). The religation efficiency is an important factor for DNA supercoiling experiments, as torque can only be applied to molecules with an intact backbone (9,10,15,45). On the contrary, our method allows also to specifically introduce a nick near the modification, by using non-phosphorylated inserts. This is required in rotor-bead experiments in which twist changes, without external torque being applied, are probed.

Our method can easily be extended to modify DNA at more than one place. Introducing repetitive BbvCI sites at different loci on a plasmid would allow to achieve their simultaneous labeling within a single preparation step. This offers great potential not only for DNA cross-bridging (41) but also for FRET experiments (46). Using multiple concatenated copies of the BbvCI-repeat sequence, even long periodic arrays of modifications may be achieved which are of interest in single-molecule experiments (46) and also DNA nanotechnology (47). Beyond introducing modifications only on one strand of the DNA, the use of BbvCI sites offers the possibility to modify also the opposite strand in a second subsequent reaction. This way for example, 4-way junctions even with additional chemical modifications can be easily inserted into long DNA constructs (7).

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