Essential genomic transactions such as DNA-damage repair and DNA replication take place on single-stranded DNA (ssDNA) or require specific single-stranded/double-stranded DNA (ssDNA/dsDNA) junctions (SSDJ). A significant challenge in single-molecule studies of DNA-protein interactions using optical trapping is the design and generation of appropriate DNA templates. In contrast to double-stranded DNA (dsDNA), only a limited toolbox is available for the generation of ssDNA constructs for optical tweezers experiments. Here, we present several kinds of DNA templates suitable for single-molecule experiments requiring segments of ssDNA of several kilobases in length. These different biotinylated dsDNA templates can be tethered between optically trapped microspheres and can, by the subsequent use of force-induced DNA melting, be converted into partial or complete ssDNA molecules. We systematically investigated the time scale and efficiency of force-induced melting at different ionic strengths for DNA molecules of different sequences and lengths. Furthermore, we quantified the impact of microspheres of different sizes on the lifetime of ssDNA tethers in optical tweezers experiments. Together, these experiments provide deeper insights into the variables that impact the production of ssDNA for single molecules studies and represent a starting point for further optimization of DNA templates that permit the investigation of protein binding and kinetics on ssDNA.
4.1 INTRODUCTION

Optical trapping is a powerful single-molecule technique that uses the gradient forces created by highly focused laser beams to trap and manipulate micron-sized particles [7]. In the last two decades, numerous highly sophisticated optical-tweezers experiments have been carried out to study fundamental cellular processes, such as DNA replication [183], transcription [70], recombination [52] and compaction [32].

These studies have revealed unexpected features of proteins interacting with DNA that were previously obscured in ensemble-averaged bulk methods. More recently, the capabilities of optical trapping have been extended by incorporating fluorescence microscopy techniques, allowing visualization and localization of individual proteins bound to a DNA molecule with nanometer accuracy, while holding the DNA at a defined tension with sub-piconewton resolution [36, 25]. The use of dual-trap optical tweezers for DNA manipulation in combination with fluorescence microscopy imposes strict requirements on the design of DNA templates: (i) the DNA molecules need to be specifically labeled (with for example biotin or digoxigenin) at their extremities in order to be attached to functionalized microspheres; (ii) the length of the DNA molecules should be 1 µm or longer to avoid polarization cross-talk between the traps [100], enhanced photo-bleaching of fluorophores [171] and oxidative attack of the DNA [39, 91]. These prerequisites have rendered the use of ssDNA in optical tweezers very difficult because of two main reasons: (i) the biochemical challenges to obtain long, end-labeled ssDNA molecules and (ii) the difficulty to tether ssDNA molecules from solution between two trapped microspheres. As a result, most of the single-molecule experiments performed on ssDNA-protein interactions made use of short oligonucleotides in combination with FRET spectroscopy [140].

Yet, in the living cell, long stretches (1000 nucleotides) of ssDNA coated with ssDNA specific proteins are present during many fundamental processes. For example, during DNA replication, the ssDNA used as a template by DNA polymerases, is often found in complex with single-stranded binding proteins [180]. In homologous recombination, recombinase proteins of the RecA/RAD51 family assemble on long segments of ssDNA and catalyse strand exchange between homologous DNA molecules [142]. Hence, “there is a need to develop methods that can image multiple pro-
teins on a long ssDNA as has been demonstrated for proteins on dsDNA” [63].

Over the last two decades, end-labeling of (long) dsDNA molecules with biotins has become the standard for single-molecule experiments, using either biotinylated oligonucleotides [154, 177, 88] or the incorporation of biotinylated nucleotides with a DNA polymerase [155]. In previous studies, it has been shown that such dsDNA constructs can be used for the generation of ssDNA templates for single-molecule experiments. To obtain the ssDNA, often a combination of force-induced melting with either formaldehyde [155] or sodium hydroxide denaturation [169] was used. Force-induced melting is described as the transition from dsDNA to ssDNA when dsDNA is kept under tension of more than 65 pN [173, 60]. When the DNA molecule is labeled on both ends of the same strand, force-induced melting can result in the release of the complementary strand, yielding ssDNA [69, 99, 155]. Alternatively, ssDNA was generated by digesting one of the strands with exonucleases [78]. However, clearing the fluidic devices of these chemicals or enzymes before experiments can be tedious. Incomplete removal of chemicals could lead to a change in pH, ionic conditions and different electrostatic properties of the glass surface, while contamination with enzymes could interfere with experiments. A systematic investigation of the parameters that impact force-induced melting as a method to generate long ssDNA templates is, however, lacking.

Maintaining ssDNA in optical tweezers for a prolonged time is more challenging than dsDNA, since the appearance of a single nick results in breaking of the molecule and the end of the experiment. Chemla et. al. have found that reactive oxygen species (ROS) created at the polystyrene surface of the microspheres due to presence of the intense trapping laser can lead to the loss of the DNA tether [91]. It was found that the mechanism of tether-loss involves the modification of the biotin-streptavidin and digoxigenin-antidigoxigenin bond linking the DNA to the trapped polystyrene bead. Use of larger microspheres moves the tethering point of DNA away from the area of ROS production and thereby reducing this issue. The impact of the presence of ROS on the actual integrity of tethered ssDNA has not been explored yet.

In this study, we describe protocols that combine, improve and generalize known methods for the labeling, melting and preservation of DNA in optical tweezers to generate reliable, long segments of ssDNA for studying
protein-ssDNA interactions. In particular, we adapted existing methods to straightforwardly label both ends of the same strand of kilobase-long dsDNA constructs of different lengths, with the option to also label one end of the complementary strand. We show that force-induced melting under medium to low ionic conditions can be reliably used to generate ss-DNA without the need of adding aggressive chemicals or enzymes. Moreover, we show that force-induced melting makes it possible to generate ssDNA/dsDNA junctions by using specific nicking enzymes. Next, we quantified methods that preserve the lifetime of ssDNA tethers kept between optically trapped microspheres. We show that the lifetime of ssDNA tethers is extended by almost an order of magnitude when we double the size of the microspheres from 2 micron to 4 micron. The easy production of ssDNA together with the increase of the lifetime of ssDNA in a tethered configuration from a few minutes to nearly half an hour opens many possibilities of addressing exciting scientific questions on processes occurring on ssDNA.

4.2 MATERIALS & METHODS

In all the following protocols, ligation is performed using T4 DNA ligase in 1x T4 DNA ligase buffer (Fermentas). Biotinylated (biotin-14-dATP and biotin-14-dCTP, Invitrogen) and non-modified nucleotides (Fermentas) are incorporated using Klenow exo- DNA polymerase (Fermentas). All modified and non-modified oligonucleotides are from Biolegio. Unless stated otherwise, restriction enzymes are from Fermentas.

4.2.1 Labeling procedure for the generation of the long ssDNA construct

The labeling of lambda DNA (see Figure 30.A) is adapted from earlier techniques to label with oligonucleotides [88]. Lambda DNA (Roche) is a linear molecule of 48,502 bp in length with two single-stranded 12-nt overhangs on both 5′-ends. To label the 3′- and 5′-end of the same strand, we used three oligonucleotides [88]. First, the 5′-end of Lambda DNA and oligonucleotide 1 and 2 were phosphorylated for 30 min at 37°C in a reaction containing 14 nM Lambda DNA or 10 µM of the oligonucleotide and 0.25 U/µl of T4 Polynucleotide Kinase in 1x T4 Ligase buffer (Fermentas).
Next, oligonucleotides 1 and 3 were annealed to the overhangs of Lambda DNA in a 10:1 oligonucleotide: DNA ratio (total volume 500 µl) by heating the reaction to 65°C for 5 minutes, after which the heat block was switched off. When the heat block was cooled down to room temperature, the ligation reaction was initiated by adding T4 DNA ligase (0.02 U/µl) and carried out for two hours. Next, oligonucleotide 2 was annealed in a 100:1 ratio to the Lambda DNA construct by incubation at 45°C for 30 minutes. As before, the reaction was slowly cooled down to room temperature by switching of the heat block. Subsequently, the oligonucleotide was ligated to the DNA at room temperature. Finally, the DNA was purified by ethanol precipitation. Ethanol precipitation was achieved by adding 1/10 volume of NaOAc (3 M, pH 5.6) and subsequently 2.5 volumes of cold 96% ethanol (-20°C). The sample was mixed by inverting 2-3 times and incubated on ice for 15 minutes. Next, the reaction was centrifuged at 12,000 g for 30 minutes at 4°C, resulting in the formation of a visible pellet of DNA. We carefully discard the supernatant without disrupting the pellet. The reaction was washed with 0.7 mL cold 70% ethanol and centrifuged for 10 minutes (12,000 g) at 4°C. Finally, the pellet was dried at room temperature and subsequently resuspended in 50 µL of 10 mM Tris-HCl pH 7.5.

4.2.2 Labeling procedure for the generation of the short ssDNA construct

Two vectors were used for the biotinylation of short dsDNA on the same strand (3’-5’ labeling): pTR19-ASDS (10,729 bp, kind gift of Dr. Y.J.M. Bollen, VU University Amsterdam) and pKYB1 (8,393 bp, New England Biolabs). In both cases, the biotinylation strategy (see Figure 30.B) followed a similar approach: on one end, biotinylated nucleotides were incorporated to a single-stranded overhang; on the other end of the same strand, a biotinylated oligonucleotide was ligated as depicted in figure 2B. To this end, the circular plasmids were first digested with EcoRI and ApaI in the case of pTR19-ASDS and EcoRI and KpnI for pKYB1 (Fermentas, FastDigest restriction enzymes). The complementary nucleotides, biotin-14-dATP and dTTP (Invitrogen), were incorporated into these overhangs. Digestion and labeling were conducted simultaneously (45 minutes at 37°C) in a reaction containing: plasmid DNA (pTR19-ASDS or pKYB1) (20 nM, in total 3 µg), biotin-14-dATP (66 µM), dTTP (66 µM), EcoRI (0.05 FDU/µl), ApaI or KpnI (0.05 FDU/µl), 0.2 units/µl Klenow exo- DNA polymerase and
**Construct type** | **Construct structure** | **Application**
--- | --- | ---
Short ssDNA molecule |  | ssDNA binding proteins
Long ssDNA molecule |  | ssDNA binding proteins<br>OT + fluorescence
Hybrid with 3’ junction | 3’ 5’ | DNA polymerase<br>Binding selectivity
Hybrid with 5’ junction | 5’ 3’ | Recombination<br>Binding selectivity

Figure 29: Schematic representation of the ssDNA constructs developed and used in this study. Red: biotinylated extremities required for the attachment to the microspheres. Black: central section available for the enzymatic reaction. Light grey: untethered section that is displaced upon force-induced DNA melting.

1x FastDigest buffer. The reaction was terminated by heat inactivation (10 minutes at 80°C). DNA was purified using ethanol precipitation, as described before. For pTR19-ASDS, the restriction digestion resulted in a 9,896 bp and an 824 bp fragment. For pKYB1, the digestion yielded an 8,356 bp and a 37 bp fragment. The 9,900 bp (pTR19-ASDS) fragment containing a 3’-overhang was separated and purified on an agarose gel using the QIAquick gel extraction kit (Qiagen). For the pKYB1 construct, the 8,356 bp fragment was purified with the QIAquick PCR Purification Kit (Qiagen). Next, biotinylated oligonucleotide 4 for pTR19-ASDS or biotinylated oligonucleotide 5 for pKYB1, was annealed to this construct (50:1 ratio oligonucleotide: DNA) by heating the reaction mixture to 65°C for 5 minutes, followed by slow cooling down to room temperature. Subsequently, the ligation reaction was initiated by adding T4 DNA ligase (0.2 U/µl). After 2 hours, the reaction was terminated by heat inactivation (10 min at 65°C) and the DNA was purified with the QIAquick PCR Purification Kit. The resulting DNA constructs are 9,900 bp or 8,360 bp long with two
biotinylated dATPs at the 3’-end, and a 25 nucleotides overhang containing 4 biotinylated dTTPs at the 5’-end of the same strand.

4.2.3 Labeling procedure for the generation of the ssDNA-dsDNA hybrid

Double stranded DNA plasmid pKYBI (8,393 bp, New England Biolabs) was digested with BaeI to create two non-complementary 3’-overhangs in a reaction containing: plasmid DNA (10 nM, 3 µg), 1x NEB buffer 4, 0.1 U/µl BaeI (New England Biolabs). The reaction was terminated by heat inactivation (20 min at 65°C) and the restricted DNA was purified using QIAquick PCR Purification kit. In the next step, two oligonucleotides were annealed to the 3’-overhangs. Depending on the desired polarity of the junction, one or both oligonucleotides were labeled with biotins on the 5’-end (see Figure 30.C). The ligation reaction was carried out at room temperature for 2 hours in a reaction containing BaeI-restricted pKYBI, oligonucleotide 1 and 2 (50:1 ratio) and 60 Weiss units of T4 DNA ligase. The reaction was terminated by heat inactivation (10 min at 65°C) and purified using the QIAquick PCR Purification kit. In the next step, the 3’-ends are filled in using Klenow exo-. For the final construct with the 5’-overhang, only one of the overhangs was filled in with biotinylated nucleotides (see Figure 30.C-left). For the final construct with the 3’-overhang, both overhangs were filled in with biotinylated nucleotides (see Figure 30.C-right). Finally the DNA was nicked either in the bottom strand (using Nb.BbvCI, New England Biolabs) or the top strand (using Nt.BbvCI, New England Biolabs). A typical reaction to create the construct containing a 6.6 kb ssDNA 5’-overhang is performed in one single step containing: 0.5 pmol of DNA (pKYBI with annealed biotinylated oligonucleotides 6 and 7), 1x Tango Buffer (Fermentas), 66 µM biotin-14-dCTP, 80 µM dATP, 0.2 units/µl Klenow exo- DNA polymerase and 0.4 units/µl Nb.BbvCI (New England Biolabs). The reaction was heat inactivated at 80°C for 20 minutes and finally purified using QIAquick PCR Purification kit.

4.2.4 Optical Tweezers setup

A detailed description of the optical tweezers instrument can be found elsewhere [59, 25]. In short, a Nd:YAG laser (3 W continuous wave, 1064 nm, Ventus 1064, Laser Quantum) was used to generate two optical traps.
<table>
<thead>
<tr>
<th>Oligo</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - Long ssDNA</td>
<td>5’- ggg cgg cga cct gga caa - 3’</td>
</tr>
<tr>
<td>2 - Long ssDNA</td>
<td>5’- agg tcg ccc ccc ttt tTt TTT - 3’</td>
</tr>
<tr>
<td>3 - Long ssDNA</td>
<td>5’- TtT tTT ttt aga gta ctt gtt tca gca tca atc ttg tcc - 3’</td>
</tr>
<tr>
<td>4 - Short ssDNA - pTR19-ASDS</td>
<td>5’- cTc TcT cTc ttc ttc ttc ttc tgg cc - 3’</td>
</tr>
<tr>
<td>5 - Short ssDNA - pKYB1</td>
<td>5’- cTc TcT cTc ttt ctc ttt ctt ctt gtac - 3’</td>
</tr>
<tr>
<td>6 - ssDNA/dsDNA</td>
<td>5’- gTg TTg gtt ttg ttg tgg gtt gat gg - 3’</td>
</tr>
<tr>
<td>7 - ssDNA/dsDNA</td>
<td>5’-TcT cTc ctc ctc ctc ttc tca agc a - 3’</td>
</tr>
</tbody>
</table>

The laser beam was split into two orthogonally polarized beams using a polarizing beam splitter cube (10BC16PC.9, Newport). The two beams were expanded with a 1:2.67 telescope system. One beam was steered by laterally displacing a telescope lens. Two traps were produced with a high-numerical aperture water-immersion objective (Plan Apo 60X, numerical aperture = 1.20, Nikon). Two microspheres (1.87 µm, streptavidin-coated, Spherotech) were held in these traps, and their microscope image was digitized using a frame-grabber board (IMAQ PCI-1409, National Instruments). We determined the distance between the two microspheres using a LabVIEW program (National Instruments), applying template-directed pattern matching. The displacement of the trapped microsphere with respect to the trap centre was detected using a position-sensitive detector (DL100-7PCBA Pacific Silicon Sensor Inc.), low-pass filtered with a cut-off frequency of 145 Hz and digitized using a 16 bit data-acquisition board (PCI 6229, National Instruments). Force and distance were simultaneously recorded by a Labview program, operating at a sampling rate of 25 Hz.

### 4.3 Results

DNA constructs suitable for optical tweezers experiments can be divided into two classes: constructs that are completely ssDNA and ssDNA/dsDNA hybrids. In the first class, dsDNA is labeled with biotins on only of the strands such that it can be converted entirely into ssDNA tethered between the trapped microspheres. Different types of ssDNA constructs are needed for different experimental configurations or biological processes under investigation. To study ssDNA–protein interactions resulting in measurable...
changes in the mechanical properties of the DNA, the use of short and thus stiffer ssDNA molecules (contour lengths shorter than about 4 µm) is advantageous to increase sensitivity [116].

On the other hand, many proteins interact much weaker with ssDNA, without producing measurable changes in the mechanical properties of the template. In this case, a general method to study ssDNA-protein interactions consists of holding a ssDNA molecule between optically trapped microspheres and simultaneously visualizing interacting fluorescently labeled proteins [176, 25, 36]. Efficient application of this approach requires long ssDNA molecules to avoid the simultaneous presence of intense trapping and fluorescence excitation lasers in the vicinity of the fluorophores [171]. We thus designed constructs with different lengths: a short ssDNA (8,393 nt and 10,729 nt) and a long ssDNA (48,502 nt) (see Figure 29). A second, different class involves DNA molecules that can be converted into
ssDNA/dsDNA hybrids, having a junction with defined polarity and position (see Figure 29). Such molecules can be used as template for characterizing substrate specificity (ssDNA or dsDNA) of DNA-binding proteins, for mimicking the physiological invading template of homologous recombination or for exploring the activity of molecular motors such as DNA polymerases, with specific activity at the ssDNA/dsDNA junction.

4.3.1 DNA labeling and generation of single-stranded DNA using Optical Tweezers

In order to generate ssDNA by force-induced melting, only one strand of the dsDNA template has to be attached to the microspheres. Biotinylation is thus performed on the 3’- and 5’-end of one of the template strands. In our approach, we use sequential annealing and ligation of biotinylated oligonucleotides to linearized DNA templates. For generation of long ssDNA constructs (48517 nucleotides), we use linearized Lambda DNA and anneal and ligate oligonucleotides (oligonucleotide 1 and 2) to first biotinylate the 5’ end of the template. A third modified oligonucleotide (oligonucleotide 3) is then hybridized and ligated to biotinylate the 3’-end of the template (see Figure 30.A). For shorter DNA constructs (8,393 nt and 10,729 nt), we first linearize DNA plasmids (e.g. pKYBI, pTR19-ASDS) using restriction enzymes to create suitable overhangs. A polymerization and digestion reaction creates a 3’-labeled end and a 3’-overhang. The final labeling is then performed by hybridizing and ligating a biotinylated oligonucleotide, (oligonucleotide 4 for pTR18-ASDS and Oligonucleotide 5 for pKYB1) in a similar fashion as for the long ssDNA construct (see Figure 30.B). To create DNA-hybrid constructs containing SDSJs with a defined polarity, we label DNA molecules on three of the 4 ends and introduced a nick at a defined position on the strand with only one labeled end (see Figure 29). Upon application of forces higher than 65 pN using optical traps, the untethered segment melts away, producing a hybrid of ssDNA and dsDNA with a defined junction. For generating this type of DNA constructs (see Figure 30.C), an oligonucleotides-based approach was used, similarly to that used for the first class of molecules. The first step consisted of the digestion of the DNA to create two non-complementary overhangs. Next, oligonucleotides were annealed and ligated to the extremities of the linearized DNA molecule, creating new 5’-overhangs. Depending on the
desired polarity (3′ or 5′) and the location of the junction (either at 20 or at 80% of the length of the DNA molecule), we used either biotinylated or non-biotinylated oligonucleotides. The new 5′-overhangs were filled in using Klenow exo-polymerase with either biotinylated or regular nucleotides. In the final step, a specific nicking enzyme was used to create a nick in the single-end-labeled strand. Finally, the constructs were tested at the single-molecule level using optical tweezers (see Figure 31).

Figure 31.: Force-extension curves of different ssDNA constructs. Black: short dsDNA construct showing the typical low-salt saw-tooth pattern in the overstretching transition. Further increase of the bead separation induces melting of the untethered segment. Red: short ssDNA construct. Green and purple curves show the ssDNA-dsDNA hybrid constructs with 5′-overhang composed respectively of 20% and 80% of ssDNA. The cyan curve corresponds to the stretching curve of Lambda DNA. The light grey curve represents the force-distance behavior of the long ssDNA construct.

4.3.2 General Applicability of force induced DNA melting

Force-induced melting[173, 60] is a crucial step in our protocol for the generation of the ssDNA constructs.
Figure 32.: Characterization of force-induced melting using the short ssDNA construct (pTR19-ASDS). [A] Force-extension measurement of dsDNA (black dots) and ssDNA (green triangles). To convert dsDNA into ssDNA, the molecule is exposed to cycles of successive straining, reannealing and probing. [B] Force-time traces at 50 mM NaCl. The extension is cycled between the straining, reannealing and probing regime, as indicated in panel A. The force drop from 65 pN to 20 pN in the second probing interval reveals that the conversion of dsDNA to ssDNA is completed within the prior 5 seconds straining interval. [C] Details of the dynamics of the final strand separation for the pTR19-ASDS DNA extended to a force of 120pN in 150mM NaCl. In the straining regime, the force drops significantly, until, after the fourth cycle, a force level of 95 pN is reached. At this force the DNA is fully melted to ssDNA, as can be seen in a force value of 20 pN in the last probing regime.

Therefore, we determined efficiency and time scale of force-induced melting for different DNA lengths at different ionic conditions. Our procedure involved three distinct steps (see Figure 32.A). First, the dsDNA was extended beyond overstretched to more than 0.58 nm per base pair (corresponding to a tension of approximately 80 pN, which we designate as the
"straining regime"). For a time $t_s$ (straining time), the dsDNA was allowed to melt. Second, the DNA was relaxed to a tension below 5 pN for a period of time (approximately 10 seconds) allowing the DNA molecules that only partially melted, to reanneal completely. After the reannealing step, the DNA has become either entirely double-stranded (when the melting process had not completed during the straining period), or single-stranded DNA (when melting had completed). Third, to probe the state of the DNA, we extended the molecule to 0.47 nm per base pair and measured the tension on the DNA. At this extension, the tension on dsDNA is 65 pN, while that on ssDNA is about 20 pN (see Figure 32) making it straightforward to discriminate between the two. We cycled multiple DNA molecules, one at a time, through such cycles of straining – reannealing - probing, until they melted completely into ssDNA, broke, or melted into an ssDNA/dsDNA hybrid.

We attribute the latter two events to the presence of nicks: breakage occurs in case the biotinylated strand is nicked; incomplete melting in case the non-biotinylated strand is nicked and only a segment of the strand is released in solution during melting. The melting efficiency (at different ionic strengths) is defined as the probability of producing a complete ssDNA tethers within a defined straining time. The melting efficiency can be therefore calculated only in experiments where complete melting take place and an intact ssDNA tether is finally produced. We restricted our analysis to only complete melting events, which amounts to approximately 30% of the $\lambda$-DNA molecules tested and about 50% of the shorter DNA tethers. This is in agreement with the nicking probability increasing with DNA length. The probability of a stable ssDNA molecule can be increased by reducing the amount of freeze-thaw cycles. Furthermore, longer dsDNA molecules, like lambda DNA, should be pipetted slowly and mixed gently, since shear forces in the liquid can potentially damage the molecule. Also, chemical impurities in the form of DNases, reactive oxygen species or other forms of mutagenic chemicals in the microfluidic system will give rise to a higher occurrence of nicks. In general, careful handling and use of freshly made DNA gives best results. Figure 32.B shows two typical probing–straining–reannealing cycles at 10 mM Tris-HCl pH 7.8 and 50mM NaCl. At the start of the experiment, the DNA was entirely double-stranded, as can be seen from the force value of 65 pN in the first probing interval. Within the next 5 seconds of straining, the molecule
completely melted into ssDNA, as is evident from the tension of only 20 pN measured in the second probing interval. For each DNA molecule showing such a tension drop in the probing period, we confirmed the complete melting into ssDNA by a complete force-extension measurement (see Figure 32). Previous single-molecule experiments have shown that the ionic strength of the buffer has a strong effect on the DNA melting process [60]. To obtain further insight in the melting process, we performed experiments at increasing salt concentrations. We observed that the dynamics of DNA melting changed significantly when raising the salt concentration. In Figure 30.C, we show an experiment similar to that in Figure 30.C, but performed at 150 mM NaCl and with a substantially higher mechanical strain applied (120 pN instead of 80 pN). In the straining regime, the force dropped significantly due to force-induced melting, until, after the fourth cycle, a force of 95 pN was reached as expected for fully melted DNA. Indeed, the DNA molecule fully melted into ssDNA, as is evident from the force of 20 pN in the successive probing cycle. To explore more systematically the impact of ionic strength, we repeated this experiment for the pTR19-ASDS DNA construct (N=121), with a constant straining force of 75 pN at different salt concentrations. We quantified our results by determining the melting efficiency, which we defined as the number of probing-straining-reannealing cycles required to fully melt all dsDNA molecules into ssDNA. This number equals one if all DNA molecules are converted to ssDNA in one cycle; less if it takes more attempts and zero if no melting occurs for this given straining time for all probed dsDNA molecules. Figure 33.A shows the melting efficiency of the pTR19-ASDS DNA construct for various straining times and ionic strengths. At 5 mM NaCl, a straining time of 1 second was sufficient to melt all probed DNA molecules. At the higher salt concentrations, the straining time had to be increased to 3 seconds (50 mM NaCl) or 5 seconds (150 mM NaCl), to obtain complete conversion into ssDNA after one cycle. Next, we increased the straining force to 110 pN to investigate the effect of DNA tension on the melting efficiency. We performed these experiments in 150 mM NaCl, using a straining time of 4 seconds to ensure that not all DNA would melt within one cycle. At a straining force of 110 pN, the melting efficiency was 0.6±0.1 (mean±standard error of the mean), higher than at 75 pN (0.4±0.1), as expected.
Figure 33: Efficiency of force-induced melting for ssDNA production. [A] Histogram of melting efficiency at different salt concentrations and straining times, using the pTR19-ASDS DNA construct exposed to a straining force of 75 pN. [B] Melting efficiency for the long dsDNA constructs at different salt concentrations and straining times. The straining force, in this case, was set to 95 pN.

We also repeated the experiment with the SDSJ and the long ssDNA constructs. For the SDSJ DNA construct, we observed a very similar melting time for the untethered segment as for the short ssDNA construct. Upon release and successive extension of the molecules, we observed a change in the mechanical properties of the tethers that are compatible with the expected ratio between single and double-stranded DNA (see Figure 31). Finally, for the long ssDNA construct, although longer straining times (10-30 seconds) and higher tensions (about 110 pN) were required (see Figure 33.B), we could melt away the untethered strand, confirming the general applicability of force-induced DNA melting for molecules of different lengths and sequences (see Figure 31).

4.3.3 Preserving the integrity of ssDNA molecules during Optical Tweezers experiments

A significant challenge in optical-tweezers experiments is to preserve the integrity of the tethered DNA to allow for measurements lasting tens of minutes. Biotin-streptavidin and digoxigenin-antidigoxigenin bonds, the commonly used strategies to attach single DNA molecules to polystyrene microspheres, can be affected by the intense 1064 nm radiation used for
the optical trapping. Two effects play a role: (i) the generation of ROS on the polystyrene surface can affect the protein-ligand interactions and (ii) the short-lived ROS can cause DNA damages, including ssDNA breaks, close to the microspheres [91, 39]. It has been shown that increasing microsphere size and lower trapping power produce more stable dsDNA tethers [91]. To test whether this approach can also be used to prolong the lifetime of the substantially more fragile optically trapped ssDNA tethers, we applied the following experimental procedure: (i) dsDNA was tethered between two optically trapped microspheres using moderate light power (500 mW per trap), (ii) force-induced melting was performed at low salt condition (10 mM Tris-HCl pH 7.6, 10 mM NaCl) to produce ssDNA, (iii) ssDNA was held at the constant tension of 20 pN until breakage occurs in the observation buffer (20 mM Tris-HCl pH 7.6, 100 mM NaCl). We found that indeed the use of polystyrene microspheres with larger diameter significantly extended the lifetime of a ssDNA molecule in optical traps: tethers between 1.87 µm diameter microspheres had an average lifetime of 3±1 minutes (mean±SD, N=71), whereas tethers between 4.26 µm diameter microspheres had a six-fold longer lifetime (18±10 minutes, mean±SD, N=33). Considering that the presence of ROS affects the bond establishment between biotin and streptavidin and not the actual bond-lifetime [91], we can conclude that the tether loss can be attributed to the single-stranded breaks generated by the presence of ROS at the polystyrene surface [91]. Furthermore, we studied the effect of the trapping light power on the lifetime of ssDNA tether in the presence of beads with large diameters (4.26 µm). Increasing the trapping power from 500 mW to 3 W per trap induced a drastic shortening of the ssDNA lifetime from 18 to 2 minutes (2±2 minutes, mean±SD, N=13). Confirming that the intensity of the trapping laser does negatively impact the lifetime of the ssDNA. Consequently, we can conclude that microspheres of larger diameter and the use of minimal trapping light result in more stable ssDNA tethers, which can be used for tens of minutes in single-molecule experiments.

4.4 DISCUSSION

Currently, many methods studying cellular processes on ssDNA, such as fluorescence polarization anisotropy, surface plasmon resonance and FRET-
based techniques, employ short homopolymeric substrates [63]. Using kilobase-long and heterogeneous sequences of ss DNA substrate mimics more closely the in vivo situation. The ability to perform single-molecule experiments on these substrates can complement, verify and extend the understanding of essential cellular processes occurring on ssDNA. Here we have provided detailed and generalized protocols for the force-induced generation of long ssDNA and SDSJ templates for optical tweezers experiments. We circumvent the many technical challenges for the manipulation of long ssDNA – the difficulty to label and form a ssDNA tether in flow - by the use of force-induced melting. First of all, we use well-established labeling techniques to label dsDNA on the same strand. Next, a tether is formed by flow-stretching dsDNA between two optically trapped microspheres and ssDNA is created using force-induced melting. By adapting this method, no addition of denaturing chemicals or exonucleases is necessary and there is no need for an extra cleaning step of the flow cell. We also report a straightforward and efficient protocol for the formation of ssDNA/dsDNA junctions constructs to study DNA polymerases or the differential binding of a protein to ss- and dsDNA. The use of a junction construct has been reported previously, where it was created by an exonuclease [78]. However, that approach did not allow the control of position and orientation of the junction, because exonuclease III only digests 400 nucleotides in the 3’- 5’ direction. By using a nicking enzyme in combination with force-induced melting, we were here able to create different oriented junctions at well-defined positions. We have also shown that force-induced melting is a generally applicable method that can be used irrespective of DNA sequence and length. In addition, we have demonstrated that force-induced melting can be employed over a broad range of salt concentrations up to the physiological range. Finally, as shown previously for dsDNA [91], the use of larger microspheres increased the lifetime of ssDNA tethers, allowing optical tweezers experiments on a single ssDNA tether for tens of minutes. We quantified that there is a six-fold lifetime increase when 4.26 µm microspheres are used instead of 1.87 µm microspheres. The protocols to generate ssDNA using optical tweezers presented here represent a convenient toolbox for reliably generating ssDNA substrates for optical-tweezers experiments without the use aggressive chemicals or enzymes. These protocols will facilitate future single-molecule experiments on a large number of genomic transactions, like the
assembly of RecA/Rad51 filaments, the binding-specificity of proteins and the dynamics of DNA polymerases.