Colloids and Surfaces B: Biointerfaces 83 (2011) 91–95

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

A new method for the covalent attachment of DNA to a surface for single-molecule studies

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ARTICLE INFO

Article history:
Received 3 August 2010
Received in revised form
27 September 2010
Accepted 1 November 2010
Available online 9 November 2010

Keywords:
DNA
Surface attachment
Covalent bond
Single-molecule
Silane-PEG-NHS

ABSTRACT

Attachments between DNA and a surface or bead are often necessary for single-molecule studies of DNA and DNA–protein interactions. In single-molecule mechanical studies using optical or magnetic tweezers, such attachments must be able to withstand the applied forces. Here we present a new method for covalently attaching DNA to a glass surface, which uses N-hydroxysuccinimide (NHS) modified PEG that is suitable for high-force single-molecule mechanical studies. A glass surface is coated with silane-PEG-NHS and DNA is covalently linked through a reaction between the NHS group and an amine modified nucleotide that has been incorporated into the DNA. After DNA attachment, non-reacted NHS groups are hydrolyzed leaving a PEG-covered surface which has the added benefit of reducing non-specific surface interactions. This method permits specific binding of the DNA to the surface through a covalent bond. At the DNA end not attached to the surface, we attach a streptavidin-coated polystyrene bead and measure force-versus-extension using an optical trap. We show that our method allows a tethered DNA molecule to be pulled through its overstretching transition (>60 pN) multiple times. We anticipate this simple yet powerful method will be useful for many researchers.

1. Introduction

Single-molecule experiments are becoming increasingly common because they allow investigators to study the distribution of biological events that are not apparent in an ensemble measurement. When conducting single-molecule mechanical studies, there are a variety of techniques to apply and measure force [1]. Methods such as optical and magnetic tweezers typically require binding of a molecule to a surface (e.g. a glass slide, bead, etc.) [2–5]. Irrespective of the type of attachment, the lifetime of the attachment bond is strongly dependent on the applied force [6]. Many measurements require large forces to probe the processes of interest including greater than 13 pN for hairpins [7], >20 pN for polymerases [8] and for nucleosome unwinding [9]. Forces of this magnitude can translate into very short lifetimes for weaker attachment bonds [6], limiting the amount of data that can be collected from a single tether. Therefore, a surface attachment strategy that creates a strong bond [10] and which allows many individual tethers to be prepared simultaneously [11] will allow for more data to be taken during one experiment.

One common way to attach DNA to a glass surface is through the non-covalent interaction of digoxigenin (dig) modified DNA with an anti-digoxigenin antibody (anti-dig) [2,12]. DNA labeled with dig at one end is bound to an anti-dig antibody that has been non-specifically adsorbed to a glass surface. This method is straightforward and binds many individual tethers to the surface, permitting multiple different measurements to be taken simultaneously [11]. The disadvantage of this method is that the dig/anti-dig interaction is not strong [13]. Once the bond is ruptured, a new tether must be found or a new sample made. Therefore, the dig/anti-dig method, though convenient, is not ideal for experiments involving higher forces. In Table 1, we compare the lifetimes of dig/anti-dig and biotin/streptavidin bonds under stress using Bell's formula [14] (Eq. (1)).

$$k(F) = k_0 e^{(FX_B/k_BT)} \quad (1)$$

Bell's formula estimates the dissociation rate at force F, $k(F)$, from the dissociation rate at zero force, k_0, the distance from the energy minimum to the rupture barrier, X_B, and thermal energy, k_BT. Therefore, the average bond lifetime is $1/k(F)$. The values for k_0 and X_B have been estimated from dynamic force spectroscopy for both the biotin/streptavidin and dig/anti-dig bonds [13,15].

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doi:10.1016/j.colsurfb.2010.11.002

Table 1
The average lifetimes of the biotin/streptavidin bond or the dig/anti-dig bonds calculated using Bell's Formula (Eq. (1)). The values used are $k_0 = 1.67 \times 10^{-5}\ s^{-1}$ and $X_B = 0.49$ nm for biotin/streptavidin [15] and $k_0 = 0.015\ s^{-1}$ and $X_B = 1.15$ nm for dig/anti-dig [13].

Force (pN)	Average lifetime (s)
Biotin/streptavidin	
10	18,000
20	5,500
30	1700
60	46
90	1.3
Dig/anti-dig	
10	4
20	0.24
30	0.015

From these estimations, it is clear that using dig/anti-dig is far from optimal for experiments requiring greater than 20 pN of force. Therefore an alternative DNA attachment strategy is to label each end of a single DNA molecule with a single molecule of biotin [9,16]. Because the biotin–streptavidin bond is much stronger than the dig/anti-dig interaction, having a biotin bound on each end allows the DNA to be subjected to higher forces with reasonable lifetimes [15,17]. However, a significant disadvantage of this technique is that, because the DNA has the same label on both ends, the DNA may wrap around so that both ends bind to the surface or bead. A technique to bind DNA between two streptavidin-coated beads is sometimes used, in which fluid flow keeps the DNA from being able to wrap around [18], however, this approach is time consuming, and only one DNA molecule may be attached at a time.

Here we present a new method of attaching DNA to a glass surface in which DNA is covalently attached to a PEG-coated glass surface via reaction of a unique terminal primary amine group on the DNA with an N-hydroxysuccinimide (NHS) group on the PEG. Fig. 1 outlines the chemistry of these reactions. This attachment strategy allows for specific binding of DNA to the surface via its amine labeled end while the other DNA end is labeled independently, permitting directionality in attachment. In the realization we present, the non-amine DNA end is labeled with biotin. The advantages of this method over previous methods are that it provides a strong bond on which to pull, there are many individual tethers on the surface, and the PEG surface provides a coating that reduces the amount of non-specific sticking [19]. Here we describe our attachment strategy in detail and show that DNA attached to a surface using this method, can be pulled through its overstretching transition multiple times with forces >60 pN. Covalent bond

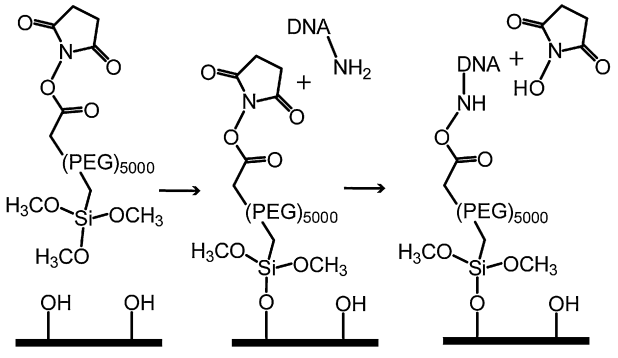

Fig. 1. Chemistry of the DNA attachment to the surface. First, silane-PEG-NHS is bound to the glass surface through the reaction of silane with surface hydroxyls. Upon addition of DNA labeled with a primary amine, the amine reacts with the NHS group on the PEG to form a covalent attachment.

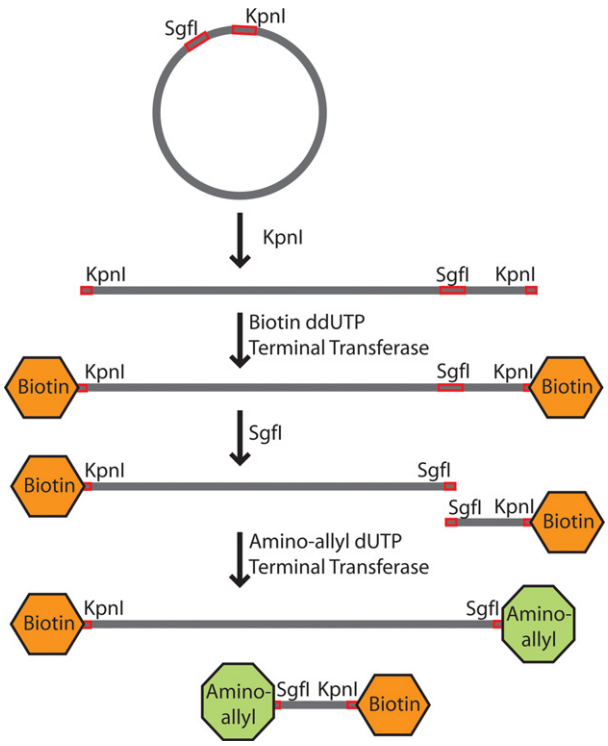

Fig. 2. A schematic of the DNA labeling strategy. A 14.5 kb plasmid is cut using KpnI, leaving 3′ overhangs. Terminal transferase is used to add biotin modified ddUTP to the free ends. The DNA is then cut with a second enzyme, SgfI, creating a 12.5 kb and 2.0 kb piece of DNA, each with a free 3′ overhang. Terminal transferase is used again to add amino-allyl modified dUTP to the free ends giving doubly labeled DNA of sizes 12.5 kb and 2.0 kb.

rupture forces are only seen with forces in the nanoNewton range [20,21].

2. Materials and methods

2.1. Labeling of DNA

Our strategy for labeling DNA, outlined in Fig. 2, was applied to a plasmid containing the restriction sites KpnI and SgfI that each yield a 3′ DNA overhang upon digestion. The plasmid was first digested using KpnI (New England BioLabs, Ipswich, MA) yielding a 14.5 kb piece of DNA. The DNA was ethanol-precipitated and resuspended in double distilled water (ddH_2O). The resulting 3′ overhangs were labeled using terminal transferase and biotin-ddUTP (Roche, Indianapolis, IN): 5× TdT Reaction buffer, 5 mM $CoCl_2$, 0.05 mM biotin-ddUTP, 400 U terminal transferase and 100 pM DNA [22]. To verify labeling, a DNA gel shift using neutravidin was performed (data not shown). The DNA was subjected to two additional rounds of ethanol-precipitation to remove salt and unincorporated biotin-ddUTP. The DNA was next digested using SgfI (Promega, Madison, WI) yielding a 12.5 kb and a 2.0 kb piece of DNA, each with 3′ overhangs. These free ends were labeled using terminal transferase and an amino-allyl-dUTP (Invitrogen, Carlsbad, CA), same as above. The DNA was ethanol-precipitated twice more to remove any unincorporated amino-allyl-dUTP. This method gives two pieces of DNA, 12.5 and 2.0 kb, each labeled with a biotin at one end and an amino-allyl group at the other. At this point, the two DNA sizes can be separated on an agarose gel and purified if desired.

2.2. Formation of flow cells

A flow cell is made by cutting a channel in 300 LSE double sided sticky tape (3M, St. Paul, MN) that is used to join a cover-

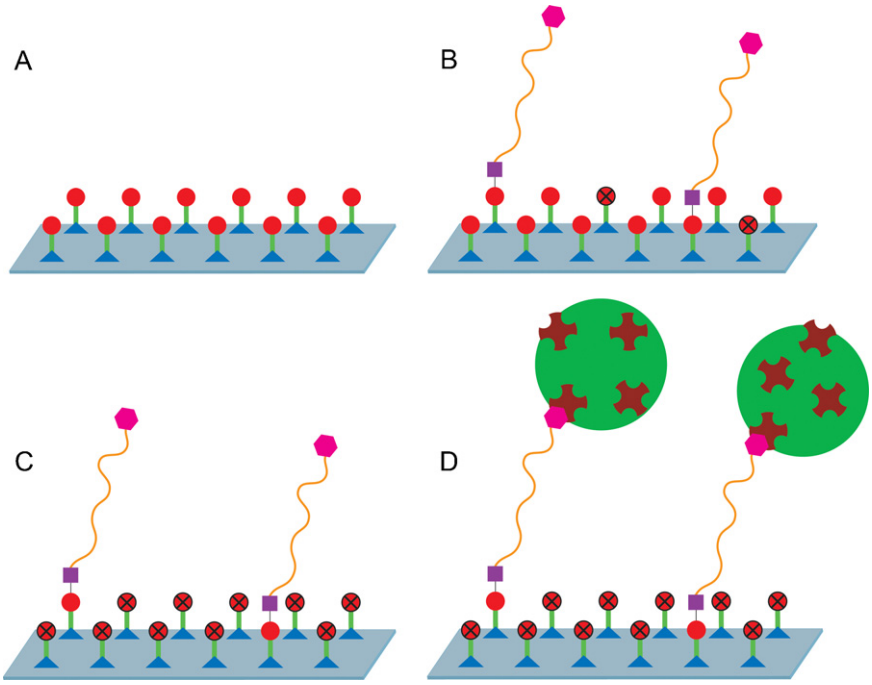

Fig. 3. Schematic of the DNA attachment method. (A) A "piranha" cleaned glass coverslip is coated with a surface of silane-PEG-NHS. The silane group (blue triangle) reacts with the glass surface, while the anhydrous DMSO preserves a reactive NHS group (red circle). (B) The DMSO is rinsed out, and DNA (red line) modified with an amino-allyl group (purple square) is flowed into the flow cell. The amine on the DNA reacts with the NHS group of the surface forming a covalent bond. The hydrolysis of the NHS by water competes with this reaction so not every NHS group reacts with DNA (hydrolyzed NHS groups are shown as black Xs). (C) Remaining NHS groups are hydrolyzed by incubation at pH 9. (D) Streptavidin coated polystyrene beads (large green spheres with red symbols) are flowed into the cell and bind to the biotin (pink hexagons) labeled end of the DNA creating a tethered particle which can be used in a variety of experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

slip and glass slide. We use the NANOPORT™ assembly (N-333, Upchurch Scientific, Oak Harbor, WA) connected over holes drilled in a microscope slide by 5-min epoxy and sealed with vacuum grease for symmetric inlets and outlets. Glass coverslips are cleaned by immersion in "piranha solution" (3:1 concentrated sulfuric acid to 30% hydrogen peroxide) for 15 min. This step serves both to remove any dirt or debris on the glass surface and to increase hydroxylation of the glass. The coverslips are then removed and placed in ddH_2O. Each coverslip is washed thoroughly with ddH_2O to remove all "piranha solution." The coverslip is then dried with a stream of nitrogen gas and immediately sealed against the glass slide/double sided sticky tape to complete the flow cell.

2.3. Application of silane-PEG-NHS surface

The silane-PEG-NHS (Nanocs, New York, NY) is dissolved in pure dry DMSO at a concentration of 1% (w/v). This solution is flowed into the flow cell and held there while the reaction is allowed to proceed for 1 h at room temperature (Fig. 3a). The dry DMSO provides an anhydrous environment that minimizes hydrolysis of the NHS groups. After an hour, the DMSO is blown out of the flow cell using nitrogen gas. The flow cell is then rinsed using ddH_2O. The flow cell is further rinsed with PBS (0.01 M phosphate buffer, 0.0027 M KCl, and 0.137 M NaCl) pH 7.3. This choice of pH serves to minimize NHS hydrolysis.

2.4. Binding DNA to the surface

To bind DNA to the surface, 100 pM of doubly labeled DNA (amino-allyl/DNA/biotin) is dissolved in PBS pH 8.3 and is flowed into the flow cell. After this point, no air should be allowed to enter the flow cell as it will destroy any tethers. The reaction of the primary amine on the DNA with the NHS group on the PEG is allowed to proceed for 1 h at room temperature (Fig. 3b). We have found that 100 pM DNA gives the optimal balance between maximizing the number of singly tethered bead attachments (approximately 10–15 in the microscope viewing area), and minimizing the number of beads tethered by two DNA molecules. However, if it is found that there are too many doubly tethered beads in a sample, the concentration of DNA can be adjusted. Next, the flow cell is then rinsed with PBS pH 8.3 to remove remaining DNA. Once the DNA is bound to the surface, any remaining NHS groups are hydrolyzed by rinsing the flow cell with pH 9.0 phosphate buffer and allowing the reaction to proceed for 1 h (Fig. 3c). This step removes any remaining active NHS groups that could react with the many primary amines on the surface of the streptavidin coated beads. If the surface is not hydrolyzed before the addition of the beads, a large number of beads stick to the surface, presumably by reaction of the NH_2 group on the streptavidin with the NHS on the PEG. Also, if the glass is not coated with silane-PEG-NHS, beads are non-specifically adsorbed to the surface and no appropriately tethered beads can be found.

2.5. Binding of the beads to the DNA

The beads, 1.26 μm diameter streptavidin-coated beads (Spherotech, Lake Forest, IL), are washed twice with PBS pH 7.3 plus 0.1% TWEEN then diluted to 0.3% mass per volume. Once the NHS groups in the flow cell have been hydrolyzed, the flow cell is thoroughly rinsed with a PBS pH 7.3 plus 0.1% TWEEN. The beads are flowed in and allowed to bind for approximately 15–20 h at room temperature (Fig. 3d). We have found that incubating for this amount of time provides the best coverage of individually tethered beads. However, incubation times as short as 1 h can generate some tethers. During this time, flow cells are stored in a humidity chamber to prevent evaporation.

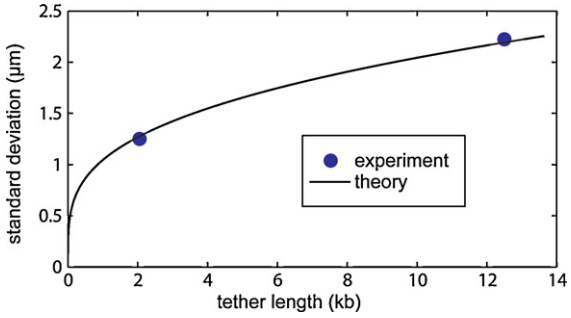

Fig. 4. A plot of the root mean square deviation of the particle from the tether point of the data (filled circles) and their comparison to calculated prediction using Ref. [24] (solid line). Error occurring from variation in measurements and deviations in bead size are ±.03 μm and ±.04 μm for the short and long tethers respectively which is too small to be seen within the size of the filled circles. The match to theory confirms that the DNA is being attached specifically via its end groups and is behaving as expected.

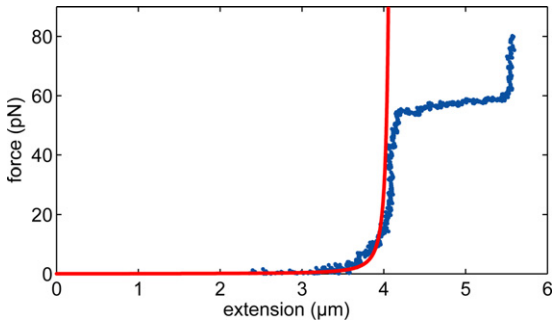

Fig. 5. A plot of the force versus extension of tethered DNA. At low forces, the force verse extension curve for DNA is well described by a worm like chain model (solid line) [12]. At forces over ∼60 pN, the DNA exhibits a signature overstretching transition, increasing in length with virtually no change in force. A single tether can be repeatedly pulled and relaxed in this fashion. In this experiment, the DNA was pulled and relaxed five times and in the final pull, shown here, the force was increased to ∼90 pN before the DNA was detached. Presumably at this point, the biotin–streptavidin bond was broken.

2.6. Single molecule manipulation

Tethered particle motion (TPM) was used to measure the length of both DNA fragments [23]. Tethered beads move as a result of Brownian motion, with the corresponding distribution of bead positions dependent on the contour length and persistence length of the tether as specified in Ref. [24]. Bead movement is recorded via video microscopy and the bead is tracked using particle tracking software [25] (see also: http://www.physics.georgetown.edu/matlab/), which resolves the position of the bead to sub-pixel resolution. The position of the bead was recorded for 100 s. The variance of the bead's position gives the length of the DNA [24,26]. A singly tethered bead was identified and centered in an optical trap in directions parallel to the surface and held 500 nm from contacting the surface. Force was applied to the DNA by moving the stage in the *x* axis, while the scatter of the trapping beam was monitored on a quadrant photo diode to measure the force [27].

3. Results and discussion

We tested the efficacy of this DNA attachment method by studying DNA structure using an optical trap. We attached one end of the DNA to a glass slide through a primary amine as described in Section 2. The other end is attached via a biotin group to a 1.26 μm diameter streptavidin-coated polystyrene bead. This setup creates a tethered bead that can be visualized and manipulated using an optical trap.

We used tethered particle motion (TPM) to measure the length of the DNA tethers [23,24,26]. Fig. 4 shows a plot of the measured root mean square deviation of the particle from the tether point. Data from seven independent measurements are averaged together for the 12.5 kb tether and from four independent measurements for the 2.0 kb tether. Each particle was monitored for 100 s at a frame rate of 5 frames per second. Also shown is the theoretical prediction for the root mean square deviation from Ref. [20], taking the DNA persistence length to be 52 nm and the bead diameter to be 1.26 μm (as per manufacturers specifications 1.26 ± .1 μm). Evidently, there is excellent agreement between our data and theory implying our attachment scheme is working as expected.

Fig. 5 shows a representative force-versus-extension curve for a 12.5 kb tether obtained at a pulling rate of 1 μm per second. For extensions up to about 3.2 μm and forces up to 60 pN, these data reproduce the force-versus-extension curve expected for B-form DNA [12]. At about 60 pN, the extension increases precipitously with little increase in force. This feature corresponds to the DNA overstretching transition [28]. Beyond an extension of about 4.6 μm, the force increases once again, corresponding to the force versus extension of overstretched DNA. In this experiment, the DNA was pulled through the overstretching transition and relaxed five times. In the final pull, shown in Fig. 5, the force was increased to 90 pN and the tether detached from the surface. The calculated lifetime of the biotin/streptavidin bond at 90 pN is around 1 s (Table 1), and presumably, therefore, the observed detachment corresponds to rupture of the biotin–streptavidin bond [15].

4. Conclusion

We have presented a new method by which to create an attachment of DNA to a glass surface that is both strong and specific. Using an optical trap, we have shown that our attachment can withstand high forces by pulling on the tethered DNA through its overstretching transition multiple times. Also, with this method, the glass surface is coated with silane-PEG-NHS, preventing non-specific sticking. Finally, this method creates many individual tethers on the surface allowing for multiple data sets to be collected from a single flow cell. The method we present is general and robust and can be used in situations where one desires strong attachment of DNA to a surface. The label incorporated on the non-amine end of the DNA can be chosen to suit the needs of a particular experiment (in our case biotin). In our experiments, we use terminal transferase to incorporate the modified nucleotides into the DNA because the DNA is very long. In other situations, however, one could readily create similarly labeled DNA using PCR with primers synthesized with modified nucleotides.

Acknowledgments

This material is based upon work supported under a National Science Foundation Graduate Research Fellowship (DS). We gratefully acknowledge funding from the Raymond and Beverly Sackler Institute for Biological, Physical and Engineering Sciences. We thank Kristina Herbert for her valuable advice and for introducing us to terminal transferase; we thank Clarence Cheng, Tijana Grove, Robielyn Ilagan, and Alice Zhou for thoughtful comments on the manuscript.

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