DNA Handles for Single Molecule Experiments

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Abstract

We have worked out a procedure for covalent binding of DNA to polystyrene microspheres for optical tweezers experiments in aqueous buffer solutions. These new coupling procedures allow preparation of DNA-microspheres in advance and storage of these beads for months. The covalent coupling of the DNA fragments facilitates the investigation of short DNA fragments (<1000 bp) and the rotationally constraint fixation of individual DNA strands with force-measuring laser tweezers. DNA-protein interactions have been investigated and the mechanical properties of individual RecA-DNA filaments have been characterized. Furthermore, DNA handles were attached to proteins to enable future probing of individual single proteins.

Introduction

Covalent attachment of DNA to a solid support is of interest for many biotechnological and single molecule applications. Recent developments improved the performance of hybridization detection by MALDI-TOF mass spectrometry [1], the spatially resolved attachment or synthesis of DNA on DNA array-based sensors [2, 3] or optical wave-guide surfaces as biosensors [4]. These preparations are focused on providing a stable covalent connection of the DNA to the solid support and the coupling reactions are realized on silicon surfaces. Another field in which immobilization chemistry has increased in importance is the single molecule manipulation using proximal probe microscopy [5 - 9], magnetic force tweezers [10] or optical tweezers [11, 12]. The fixation of biomolecules tethered between movable surfaces is essential during studies where the mechanics of single molecules is explored. In force spectroscopy experiments, where the mechanics of long biomolecules is investigated, an unspecific anchoring of the molecule on the surfaces is applicable. The exertion of a high loading force (>1 nN) during the contact of the surfaces is enabling the unspecific attachment of the molecule on the surfaces [8]. However the use of short molecules requires a specific coupling of the molecules on the surface and in addition the introduction of a spacer molecule to avoid masking of the signal due to nonspecific interactions [6]. The surfaces involved in these experiments are silicon and noble metals. Magnetic tweezers and optical tweezers probed long polymers such as λ-DNA [10, 13, 14] or proteins that naturally occur as tandem arrays of globular domains [15]. The molecules of interest are linked to commercially available polystyrene microspheres with or without magnetic core. The binding to the surface was accomplished by interacting attached ligands (e.g. Biotin, Digoxigenin, Antigens) on the ends of the polymers with receptor molecules on the surface of the beads (e.g. Streptavidin, Avidin, Antidigoxigenin, Antibody). The use of these instruments doesn't allow an unspecific attachment as in the case of the proximal probes due to the fact that loading forces are limited to below 200 pN. We developed a method to covalently couple DNA directly to polystyrene microspheres for a permanent and oriented fixation of short molecules. This paper gives a detailed description of the procedure, which is also a part of a paper upon single DNA-RecA filament polymerization and mechanics by Hegner et

al. (1999). In this work we focus on single molecule coupling to solid surfaces and show some applications of single molecule manipulation. In addition we introduced DNA handles attached to protein molecules to enable future investigations on a single molecule level.
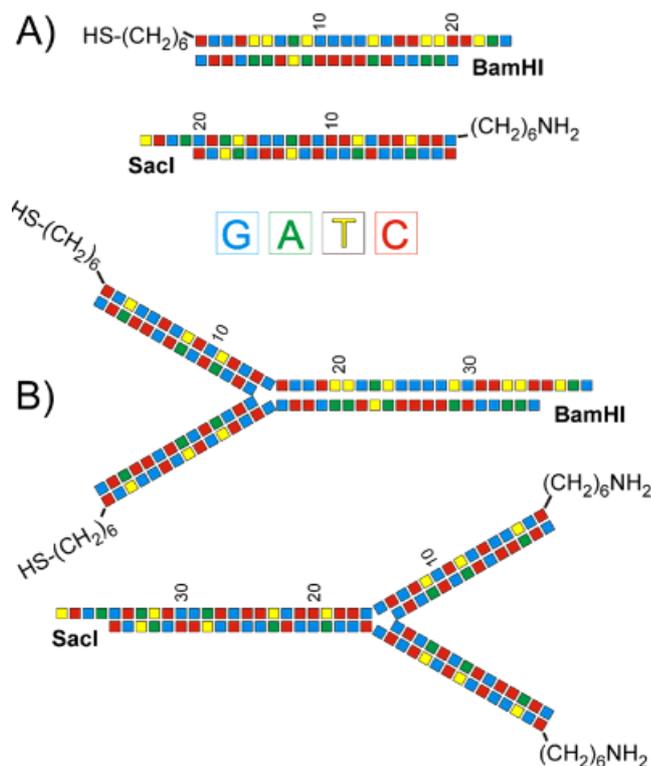

Fig. 1. *Design of the DNA oligonucleotides. Ligation to dsDNA fragments through BamHI respectively SacI overhang restriction sites. A) Covalent coupling to both surfaces trough one strand of the DNA. B) Torsionally constraint covalent coupling of two ends to the surfaces.*

Experimental

Modification of DNA

A 10417 bp respectively a 4000 bp fragment was cut from a 14417 bp supercoiled DNA plasmid using 2 restriction enzymes, which left different 4-base overhangs. Small double-stranded DNA (dsDNA) linkers subsequently modified the ends of these DNA fragments. First it was necessary to check the quality of modification of these commercially available oligonucleotides since the quantity of actually modified end-groups varied greatly from batch to batch. The single stranded DNA (ssDNA) linkers were then

phosphorylated with T4 polynucleotide kinase (NEB Inc., Berverly, MA) and annealed in 100mM NaCl, 1 mM HEPES pH 7.0. This resulted in two short dsDNA linkers (20 bp each) with complementary overhangs or dsDNA linkers (35 bp each) with complementary overhangs and Y-shaped ends (see Fig.1). The dsDNA linkers had different chemical modifications on their outboard ends, one end being thiol-modified on the 5' blunt end while the other end was amino-modified on the 3' blunt end, yielding dsDNA modified on both ends of the same strand. Either modification can be exchanged with any terminus-modifier available for oligonucleotide-synthesis (Glen Research, Sterling, VA). These annealed dsDNA linkers were then used for the subsequent modification. The ligation of these oligos to the ends of the DNA fragment was carried out in a ratio of $\geq$ 250 oligos per DNA end using < 5 nM long DNA in 100mM NaCl, ligation buffer (NEB) at 4 °C. After the ligation the DNA was purified and cut blind out of a conventional agarose gel. Exposure of the DNA to ethidium bromide and UV light was omitted since this would induce nicks in the DNA, which is undesired in the single molecule experiments. Thereafter the DNA was extracted by electroelution (Elutrap, Schleicher&Schuell, Germany) from the agarose without any precipitation step at the end of the extraction. The electroelution buffer was exchanged to 1 mM HEPES pH 7.0 in Microcon filters (Amicon/Millipore, San Jose, CA). The DNA could then be stored at 4°C.

Activation of Polystyrene Microspheres

The surface of carboxyl- or amino-functionalized polystyrene beads (Bangs Laboratories, Fishers IN; Spherotech Inc. Libertyville, IL; Interfacial Dynamics Co. Portland, OR) was activated with heterobifunctional crosslinkers [i.e. 1-Ethyl-3-(3-Dimethylaminopropyl) carbodiimide Hydrochloride (i.e. EDC) and N-Hydroxysulfosuccinimide (i.e. sulfo-NHS) or Sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (i.e. sulfo-SMCC) (Pierce, Rockford, IL)]. The concentration of the functional groups (i.e. -COOH or -NH$_2$) was calculated according the numbers provided by the manufacturer (i.e. surface charge density (parking area/group), surface area/bead, number of microspheres/ml). A 1000 fold concentration of sulfo-SMCC relative to the concentration of the functional groups on amino modified polystyrene beads was incubated in 20 mM HEPES at pH 7.5 for 1 hour on a shaker at room temperature (20 – 25° C (RT)) which resulted in thiol-reactive microspheres. Carboxy-modified microspheres were activated with EDC and sulfo-NHS with a 1000- respectively 5000-fold concentration relative to the surface group concentration in 50 mM MES at pH 6.0 for 1 hour on a shaker at RT (amino-reactive beads). Thereafter the beads were extensively washed within < 30 minutes by successive

precipitation of the beads at 4 °C with ice-cold 1mM MES pH 6.0 to drop the free crosslinker concentration below 10 fM.

Covalent Coupling to the Activated Beads

The reactivity of the beads was checked by coupling ^{35}S-cysteine to the surface. In the first experiment the total incubation time was varied. In a second experiment the stability of the activated beads was checked. After the activation of the beads was completed individual batches were incubated for 30 minutes varying the start point of the incubation. The overall concentration of the cysteine was adjusted 1:1000 with cold carrier cysteine to equal the concentration of the activated surface groups on the microspheres. The ^{35}S-cysteine coupling was performed in 100 mM NaCl, 20 mM HEPES at pH 7.0, 7.5 and 8.0. The coupling reaction was stopped by adding 20 mM β-mercaptoethanol when sulfo-SMCC was used, respectively with 20 mM ethanolamine when EDC-and sulfo-NHS were used as activation agents. The modified beads were separated from the supernatant by precipitation. The coupling efficiency then was checked by measuring the radioactivity of the resuspended bead pellet and the supernatant in a scintillation counter.

The modified DNA was coupled to the activated beads the following way: After the washing cycles the bead pellet was resuspended in coupling buffer [i.e. 50 mM HEPES, 500 mM NaCl, 1 mM EDTA, 50 μM TECEP (Pierce, Rockford, IL) at pH 7.0 for the sulfhydryl coupling and at pH 7.5 for amino coupling]. Individual double stranded DNA molecules were then immediately coupled to the thiol- respectively amino-reactive polystyrene microspheres by incubation of 2000 - 4000 DNA's per bead for 3 hours at RT in coupling buffer. The volume of the coupling reaction was chosen to have a $\geq$5% volume of matter (DNA and beads) per reaction volume. The volume of the DNA was calculated to be the volume of a sphere of a random coil with a radius of gyration $<R_G^2> = <h^2>/6$ and $<h^2> = 2PL(1-P/L(1-e^{-L/P}))$ with P being the persistence length (i.e. 53 nm for B-DNA [13]) and L being the contour length of the DNA fragment [16]. After completed reaction the DNA-beads could be stored at 4°C and were stable for months.

DNA-Protein Interaction in Optical Tweezers

For DNA-protein interaction experiments with optical tweezers one end was chosen to be biotinylated while the other end was covalently coupled to the beads according to the methods described in the previous section. A polystyrene bead with the coupled DNA was held in the laser trap, while the other end of the molecule was attached by a streptavidin-biotin linkage to a polystyrene bead held at the end of a micropipette by suction. The center-to-center distance between the beads was measured by capturing the beads' images in a video frame-grabber. Moving the pipette relative to the trap extended the DNA molecule. Reference force-extension curves for single dsDNA or ssDNA–molecules were obtained in assembly buffer [33 mM HEPES pH 7.2, 50 mM NaCl, 5 mM MgOAc]. A solution of 2 mM RecA (New England Biolabs) in polymerization buffer [33 mM HEPES pH 7.2/100 mg ml-1 BSA/50 mM NaCl/5 mM MgOAc/2 mM DTT/1.5 mM nucleotide cofactor] was flowed through the fluid chamber when RecA-DNA filaments were studied on either dsDNA or ssDNA molecules. All experiments were performed at RT.

Atomic Force Microscopy (AFM) data were taken with a commercial instrument (Nanoscope IIIa-Multimode AFM, Veeco-Digital Instruments, USA). Images were taken under ambient conditions with AFM silicon cantilevers (Nanoprobe, Wetzlar, Germany) in tapping mode operation.

Results and Discussion

Modification of the DNA

The DNA was modified in two different ways. The first type of modification introduced an alkyl spacer of $(CH_2)_6$ and a reactive group (e.g. HS, NH_2 or biotin) on the 5' respectively on the 3' end of the 20 bp ssDNA yielding a dsDNA modified on both ends of the same strand after the ligation step. The second type used 35 bp ssDNA oligos and two additional 14 bp oligonucleotides. They were annealed simultaneously and form an Y-shaped end with a hinge on both ends of the 35 bp dsDNA linker (see Fig. 1). This modification was introduced to enlarge the radius for the coupling of the second end on the same side to the surface of the beads in the case where a torsionally constraint coupling was required. The radius of one thiol-alkyl spacer is calculated to be the Flory radius being $R \sim P*N^{0.6}$ [17] where P is the persistence length taken here with $P \sim 0.15$ nm and N = 7, one finds $R \approx 0.48$ nm. The area A, which two of these spacers on one end of the dsDNA explore is $A \approx 4\pi R^2$ being ~ 2.90 nm^2. Using the Y-shaped dsDNA linkers increases the individual radius of one arm to $R \sim L*N$, where L is the length of one bp $L \sim 0.34$ nm. Since the 14 bp are shorter than the persistence length of the DNA the arm is a stiff object. This results in a rotationally free arm of ~4.7 nm per 14 bp and adding the alkyl spacer results in a length of ~ 5.2 nm. If the arm is allowed to freely rotate the area which can be explored by two of these objects is then $A \sim 340$ nm^2. This increases the chance that both endgroups bind to the surface. The parking area per active group on these highly modified polystyrene microspheres is in the order of ~ 0.8 nm^2 and probably not all the groups are being activated during the activation reaction.
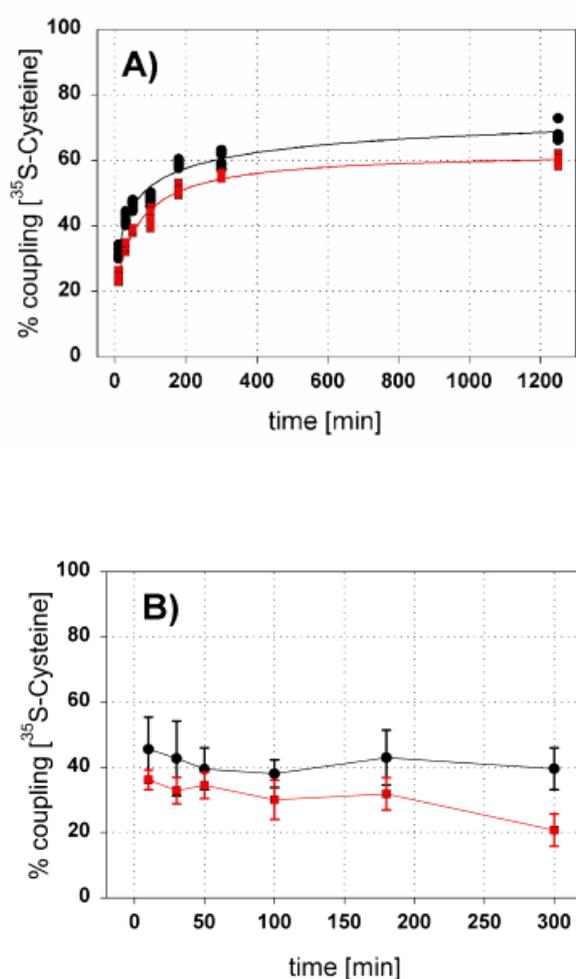
Fig. 2. *Reactivity of the activated polystyrene microspheres. Black symbols coupling of sulfhydryl group of ^{35}S-cysteine to sulfo-SMCC activated polystyrene beads at pH 7.0, red symbols coupling of amino group of ^{35}S-cysteine to EDC / sulfo-NHS activated beads at pH 7.5. A simple power law function was fitted to the experimental points and is shown as guidance for the eye. A) Amount of coupled ^{35}S-cysteine to microspheres upon varying the total incubation time. B) Stability of the activated beads during one working day probed by coupling ^{35}S-cysteine for 30 minutes starting the coupling reaction after certain intervals after the activation.*

Activity of the Polystyrene Beads

Polystyrene microspheres with a size of ~ 3 mm diameter were activated, using an excess of heterobifunctional crosslinkers, to make them reactive towards thiol groups respectively amino groups. In order to gain insight into the reactivity and the stability of the activated polystyrene microspheres, the beads were incubated with radioactively

labeled ^{35}S-cysteine. The reaction seemed to have an optimal speed and stability by incubating thiol labeled substances at a pH 7.0 whereas amino modified substances were coupled at a pH of 7.5. In figure 2A the total amount of coupled ^{35}S-cysteine on the bead is shown. The coupling of the amino acid as a test substance showed that half of the capacity of the coupling reactions is reached after 30 - 40 minutes whereas incubation for longer than 3 hours yielded in a neglectable increase of additionally coupled material. A concern during these experiments was that the activated beads would show a rapid decay of the reactivity towards their targets. We then incubated the labeled amino acid for a limited time and observed the amount of bound material starting the incubation of each set after certain time intervals.

Figure 2B shows the stability of the activated beads during a few hours. As shown the reactivity of the maleinimid group towards the thiol group didn't decay much working at a pH 7.0. The amount of thiol groups coupled to the surface didn't change much varying the pH from 7.0 to 8.0. The fact that the reaction of maleinimid groups with sulfhydryls proceeds at pH 7.0 at a rate 1000 times faster than its reaction with amines let us choose pH 7.0 as standard reaction condition [18]. The coupling of amino groups to the EDC / sulfo-NHS at a pH 7.5 already showed a decrease of coupled material to the surface after three hours. In a different work where the reactive NHS groups were tightly packed on an activated surface for biomolecule coupling a decay of coupling activity is not visible during more than 18 hours up to pH 8.5 [19]. This lead to the conclusion that some of the active groups hydrolyzed and lost their activity [18]. pH 7.5 was chosen as standard condition to increase the amount of non-protonated amino groups for crosslinking and still have a reasonable coupling efficiency and stability.

For the single molecule experiments with optical tweezers it is desired to have in average one functional DNA fragment coupled per bead. When DNA was coupled to the surfaces a surplus of 2000 - 4000 modified DNA's per bead had to be used. The high amount necessary during the coupling reaction was probably caused through the low amount of actually modified end groups per DNA and the slower diffusion of the long DNA fragments in the solution compared to the amino acids in the coupling reaction assays.

Force Spectroscopy

Single molecules of double-stranded DNA were stretched with force-measuring laser tweezers. In the performed optical tweezers experiments one end of the DNA was coupled covalently to the bead surface whereas the other end was labeled with biotin. The biotinylated DNA end could then be linked to a streptavidin or avidin coated bead on a micropipette in the fluid chamber of the instrument. It was possible to covalently couple the second end of the DNA in

situ to chemically activated microspheres. The experimental throughput was not as high as in the case were one DNA end was biotinylated. In such a setup the activated beads to couple the second end have to be prepared fresh for every experimental day. In figure 3 typical force-extension data from bare DNA and from a ssDNA-RecA filament measured by optical tweezers are shown. The figure shows the same molecule in three different forms. The bare dsDNA was pulled (blue circles) showing the characteristic reversible overstretch force plateau at 68 pN [13]; this dsDNA was then converted to ssDNA by exposing the dsDNA to one high force cycle [8, 11] (force >120 pN, see asterix in Fig.3). This process released the unmodified strand into solution, yielding a naked ssDNA (green squares). To investigate the interaction of RecA proteins with DNA in the subsequent step the molecule was complexed with RecA-ATP[γS] monomers, forming a RecA-DNA filament (red triangles).
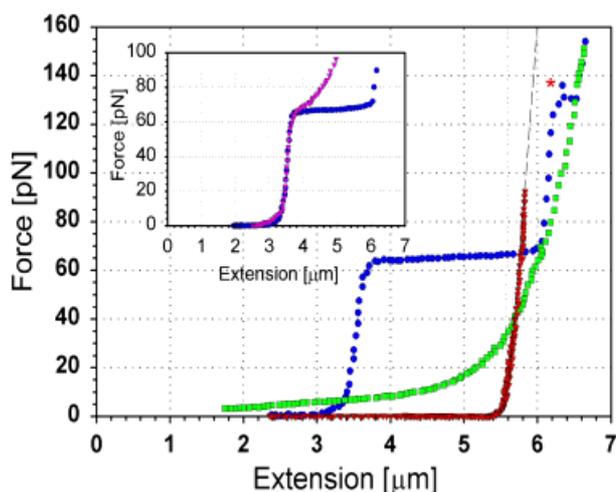
whether one or two DNA chain were present in its core. The stretch modulus of dsDNA in the filament was nearly twice that of ssDNA. The fact that the two strands contributed independently to the stiffness of the filament suggested that each DNA strand adopted a similar structure without affecting the structure of the other strand [11].

When both strands at each end of the molecule were attached to both beads using the Y-shaped oligonucleotides, the DNA was torsionally constrained. Since the second bead in the optical trap was allowed to freely rotate no additional twisting could be induced and the DNA was torsionally relaxed. But when being pulled no overstretching plateau to S-form DNA was observed (see inset figure 3). The DNA showed a steep increase at forces higher than the overstretch plateau of freely swiveling DNA fragments, comparable to the data presented by Leger et al (1999).
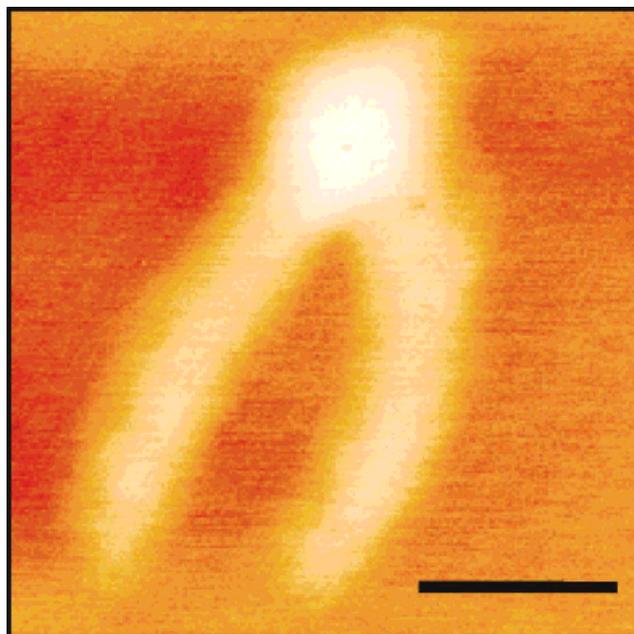

Fig. 3. *Force vs. extension plots measured by optical tweezers. The blue curve (circles) shows the force vs. extension for bare dsDNA, and the green curve (squares) shows the bare ssDNA in assembly buffer. To convert the dsDNA to ssDNA the dsDNA was pulled to forces higher than 120 pN (asterisk). At this force the second strand was released into the surrounding assembly buffer. A ssDNA-RecA filament is shown in red triangles. The dashed curve shows the extensible WLC model with contour length ~ 5.6 mm and a persistence length of ~ 900 nm including a stretch modulus of ~2200 pN. (Inset) Force vs. extension plot for bare dsDNA freely swiveling around its ends anchored through one strand only (blue circles) and for bare dsDNA being torsionally fixed through two both strands on both beads (pink triangles).*

Fig. 4. *Single protein molecule modified with molecular handles of 150 bp dsDNA measured by AFM under ambient conditions. Bar 20 nm (Molecules: courtesy of A.A. Deniz, Scripps Research Institute, La Jolla, CA.)*

Conclusions and Perspectives

The site-specific coupling of DNA via covalent bond to polystyrene microspheres allows the investigation of short DNA fragments with optical tweezers. The universal design of the linkers enables the ligation to any DNA fragments as long as the overhanging bases were adjusted to the restriction sites. The covalent anchoring to both surfaces also facilitates the investigation of single molecules at thermal equilibrium at various loading forces without

Both dsDNA- and ssDNA-RecA filaments were formed in polymerization buffer and were very stable. The filaments could be stretched with forces up to 150 pN without changing their characteristics. The persistence length of the filaments was in the range of ~ 900 nm and insensitive to

releasing the molecule into the solution. When manipulating single globular proteins or tertiary folded RNAs, additional problems must be overcome. As a result of their small dimensions (typically 1.5 - 6 nm) direct tethering to the surfaces is undesired. Furthermore, the non-specific interactions of the surrounding surfaces would mask the signal of the small individual molecule. These interactions, and possible solvent exclusion within the gap, could lead to denaturation of the molecule or perturbation in the force profiles. Methodology has been developed with Deniz and Schultz [21] to overcome these problems by site-specifically introducing stiff DNA handles to extend the dimensions of the protein/RNA molecule. This approach truly allows the probing of individuals rather than of arrays of smaller molecules, providing a way to avoid the convolution inherent in experiments on arrays. An example of 150 bp dsDNA handles site-specifically and covalently attached to a T4-lysozyme protein molecule is shown in figure 4, clearly demonstrating the potential of the methodology. A covalent attachment of these DNA-protein hybrid molecules to surfaces via the outer ends of the DNA handles minimizes the non-specific interaction of the surfaces with the protein/RNA molecule, and would allow probing the single protein molecules. A DNA handle length of ~ 60-100 bp on each side would be sufficiently stiff and hold the protein/RNA 20 - 34 nm from the surface, allowing room for free exchange of ligands or solution in the environment of the molecule.

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