Assembly of DNA Rotaxanes for AFM Based Sequencing

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ABSTRACT

Development of DNA sequencing technologies has been fueled by the desire to unlock the genetic information stored inside of genomes. This information can help fields as varied as molecular medicine, bioarcheology, forensics, and environmental science. The challenge is to establish a method that is more cost effective and faster than the ones currently available. Our technique takes advantage of the atomic force microscope's (AFM) ability to detect piconewton scale forces in order to read off sequencing information based on the interaction of nucleotides with a cyclodextrin.

Keywords: rotaxanes, DNA, AFM, sequencing

1 INTRODUCTION

The atomic force microscope's (AFM) ability to sense piconewton forces with single molecule systems makes it a powerful tool for analyzing interactions at the molecular level [1]. We are building a system for sequencing single strands of DNA based on the AFM force measurement in conjugation with the supramolecular chemistry. Our set-up relies on a ring molecule, covalently bonded to and pulled by an AFM cantilever, sliding over a strand of DNA tethered at one end to a non-adhesive surface (see Figure 1). Interactions between the DNA bases and the ring molecule can be distinguished by the size, dipole moment, and hydrogen bonding of the different bases and force extension curves will show these differences. Thus, a DNA sequence can be read out by analysis of the force curves.

A prerequisite for the AFM based DNA sequencing is to form a rotaxane composed of the ring molecule and a DNA conjugate. The complex formed when a guest or leading molecule threads a host molecule, in this case a cyclodextrin ring, is a pseudo-rotaxane. If either end of the leading molecule is stoppered, so the cyclodextrin can not slide off, it becomes a rotaxane. The rotaxane formation takes advantage of the hydrophobic interactions between threading molecules and cyclodextrins in aqueous solutions. Cyclodextrins consist of glucose molecules linked together to form a toroid shape [2]. The exterior of a cyclodextrin is hydrophilic and it easily dissolves in water. The interior is hydrophobic and when it is mixed with a linear organic molecule in the aqueous solution, the molecule will spontaneously thread the cyclodextrin [3].

We are investigating two methods of rotaxane formation. One is assembled in solution using a twelve carbon alkane terminated with an amine group at one end as the leading molecule. The other end of the alkane is attached to single stranded DNA to be sequenced. Carboxyfluorescein *N*-succinimidyl ester is adapted as a stopper bound to the amine group. The other method builds the rotaxane on an adamantane functionalized surface.

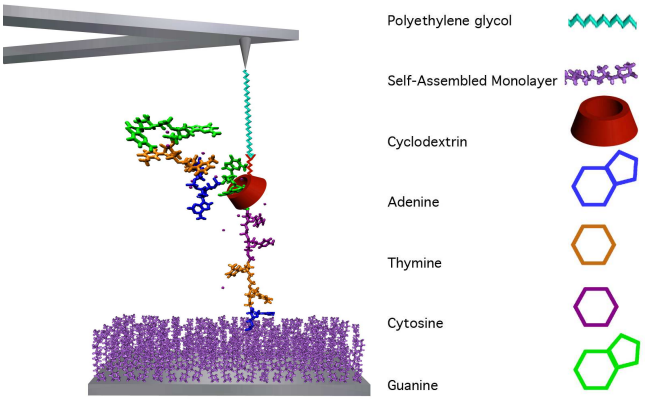

Figure 1: Schematic of DNA sequencing by AFM.

2 EXPERIMENTAL SECTION

Modified DNA was acquired from Integrated DNA Technologies (Skokie, IL). Three sequences were used; a 15mer poly thymine and 5'-ACT GAC TGA CTG ACT with an amine functionalized 12 carbon chain at the 5' end, and 5'-$(CCC)_4(AAA)_4(CCC)_2$CCA AAA CCA ACC AAC A with a protected thiol at the 5' end and Texas Red at the 3' end. β-Cyclodextrin was obtained as a free gift from Cerestar (Mechelen, Belgium). The acetonitrile used for purification was HPLC grade from Pierce (Rockford, IL). Silanes, n-propyldimethylchlorosilane (n-propyl silane), and 3-Aminopropyldimethylethoxysilane (APDM) were purchased from Gelest (Morrisville, PA) and used neat. Vinyl Sulfone PEG NHS 3200(Daltons) was purchased from Nektar Therapeutics. PBS (pH 7) buffer was purchased from VWR (West Chester, PA). All other chemicals were purchased from Sigma-Aldrich. All water was 18 mΩ from Nanopure Diamond of Barnstead. We

used a UV Clean #135500 from Boekel Inc. FTIR spectra was recorded on Nicolet 6700 spectrometer from Thermo Electron and mass spectrometry was done on a Voyager DE STR MALDI-TOF mass spectrometer. Varian Inova 500 NMR was used for all two dimensional NMR spectra. The AFM was a Pico Plus from Molecular Imaging (Tempe, AZ). Cantilever tips were ultra sharp csc11 tips purchased from Mikromasch.

Silicon wafers were cut into centimeter squares. The wafers and cantilevers were then ozone cleaned for ten minutes. They were immediately plunged into piranha for one minute for the wafers and 30 seconds for the cantilevers. The surfaces were immediately rinsed with water for 30 seconds and put into 1 ml ethanol containing 5 µL APDM and 200µL n-propyl silane for 5 minutes. The tips were immediately rinsed with water and placed in 1 ml ethanol and 200 µL APDM for 5 minutes. Both the tips and the wafers were rinsed in water and then put into a clean desiccator, backfilled with argon and vacuumed out to cure for one hour.

The procedure for the synthesis of 4,4'-bis(6-hydroxyhexyloxy)biphenyl was taken from Bagheri *et al* [4]. Synthesis of 4,4'-bis(tripropylene glycol)biphenyl was taken from Cordova *et al* [5].

2.1 NMR Study of Pseudo-Rotaxanes

A 1:1 ratio mixture of 2,6-di-O-methyl-β-cyclodextrin (2mg, 1.4µmol) and 1,12-diaminododecane (0.3mg, 1.4µmol) in D_2O was stirred overnight, filtered and used for the NMR study. A COSY (Correlated SpectroscopY) in conjunction with HMBC (Heteronuclear Multiple Bond Correlation) and HMQC (Heteronuclear Multiple Quantum Coherence) were used to assign the hydrogens of the cyclodextrin. The ROESY (Rotational Overhauser Effect SpectroscopY) was used to identify the interactions between the cyclodextrin and threading molecules.

2.2 Synthesis of DNA Rotaxanes

The first step in the formation of the solution phase DNA rotaxane was to mix the modified DNA (0.12mM) with β-cyclodextrin (0.028mg, 0.02µmol) in a 1:2 ratio in pH 7.5, 50mM phosphate buffer overnight. . An excess of 6-carboxyfluorescein *N*-succinimidyl ester (0.05mg, 0.12µmol) was added after being dissolved in dimethyl sulfoxide to complete the rotaxane (see Figure 2). The rotaxane was purified on an Agilent 1100 series binary pump system. A gradient of 0-30% acetonitrile with 100mM fresh triethylammonium acetate buffer was used for separation with the detector set at 260nm and 500nm. Matrix-assisted Laser Desorption/Ionization was used to verify the formation of the rotaxane.

2.3 Assembly of DNA Rotaxanes on a Surface

To form the rotaxane 1,3-adamantane (25mg, 0.1mmol) diacetic acid, N, N'-dicyclohexylcarbodiimide (80mg, 0.4mmol), N-hydroxysuccinimide (11mg, 0.1mmol) were added to DMF (1ml) and stirred for 15 minutes. The wafers were then added to this solution and stirred for 1 hour and rinsed with water. Then they were placed in a solution of N, N'-dicyclohexylcarbodiimide (80mg, 0.4mmol) and N-hydroxysuccinimide (11mg, 0.1mmol) for 10 minutes, and then rinsed with DMF and transferred to 1,12-Diaminododecane (20mg, 0.1mmol) in DMF (1ml). The wafers were allowed to sit for 10 minutes and then rinsed well with water. The wafers were then placed in a solution of carboxymethyl-β-cyclodextrin (107mg, 0.07mmol) in pH 7 PBS buffer (1ml). After 20 minutes the VS-PEG-NHS (30mg, 0.09mmol) was added to the solution and stirred for one hour. After the hour the surfaces were rinsed well with water. DNA deprotected with tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (0.02%) was incubated on the wafer and allowed to react for 1 hour. The wafers was rinsed and then placed in a solution of *N*-ethyl-*N*'-(3-dimethylaminopropyl) carbodiimide (20µl ,0.012mmol), and cystamine (7mg, 0.012mmol) in pH 7 PBS (10ml) for 15 minutes (see Figure 3). The tips were removed from the vacuum and placed in pH 7 PBS buffer (100µl) with VS-PEG-NHS (45mg, 0.01mmol) for 15 minutes. They were then rinsed and used immediately.

2.4 IR Study of Surface Bound Rotaxanes

Using Grazing Attenuated Total Reflectance (GATR) IR various surfaces were analyzed for the presence of cyclodextrin and the assembly of rotaxanes. The first surface served as a control with cyclodextrin dissolved in water, deposited on a silicon wafer, and allowed to dry. A surface was prepared as described above with adamantane, and cyclodextrin incubated for 20min. The surface was then rinsed with water. The final surface tested included the PEG linker used to trap the cyclodextrin on the surface.

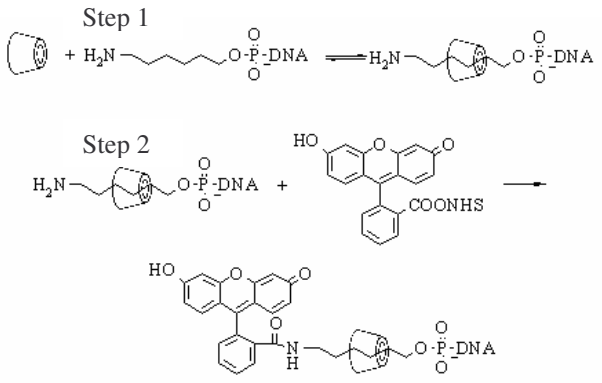

Figure 2: Formation of the pseudo-rotaxane, step 1, and the rotaxane, step 2.

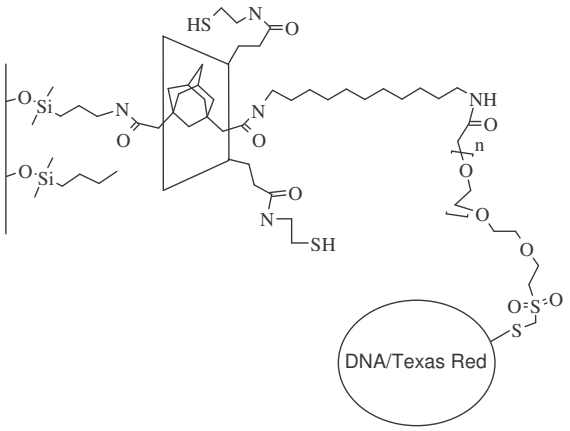

Figure 3: Surface bound DNA rotaxane

3 RESULTS AND DISCUSSION

We have observed that single stranded DNA molecules cannot form rotaxanes with cyclodextrin. The primary work was done to find threading molecule suitable for formation of the DNA rotaxanes. Based on a solubility and NMR study of six molecules and modifications 12-carbon alkyl chain was chosen as a thread (see Table 1). A conjugate of the amine terminated 12 carbon alkyl chain with DNA was synthesized for the study. The positively charged amine at the end of the carbon chain is utilized to stabilize threading under the neutral condition [6]. Accurate chemical shifts of the solitary cyclodextrin ring were assigned. It was found that the hydrogens in the interior of the ring (H3 and H5) have chemical shifts of 3.89ppm and 3.76ppm, respectively. Once that information was known the leading molecule was added and a ROSEY was taken, Figure 5. The red rectangles highlight the peaks corresponding to the hydrogens of 1,12-diaminododecane (1.28ppm) being close in space to H3 and H5 of 2,6-di-O-methyl-β-cyclodextrin, which confirmed that 1,12-diaminododecane and a cyclodextrin ring form a pseudo-rotaxane. The next step was to add the DNA conjugate. The formation of the DNA rotaxane was verified by MALDI. Once the rotaxane was formed it was purified on the HPLC. The fractions were checked for the rotaxane with MALDI and integration of the appropriate peak showed a 14% yield (see Figure 4). Control experiments were also preformed with DNA that did not include the carbon chain. No threading occurred.

The assembly of surface bound rotaxanes was verified using FTIR (see Figure 6). The red trace shows the fingerprint region of cyclodextrin dried on a glass surface. The magenta trace was recorded after the cyclodextrin was washed away from the complex with adamantane without the PEG stopper. When the stopper is added, the green trace, then the cyclodextrin remains on the surface. Pulling experiments were preformed with the surface prepared rotaxane. Texas Red dye at the end of the DNA was used to catch the sliding cyclodextrin to result in DNA extension. The unprotected sulfur of the cystamine was added to the cyclodextrin so it could bind to the maleimide coated AFM tip. Figure 7 shows a sample of the force curves which follow worm like chain behavior [7]. Control experiments without any cyclodextrin were also carried out and show no extension curves.

Guest Molecule	β-CD		Dm-β-CD	
	S.	NMR	S.	NMR
Biphenyl-4,4'-diol	S	T	S	
2,2'-Dimethylbiphenyl -4,4'-diol	NS		S	T
4,4'-bis(6-hydroxy hexyloxy)biphenyl	NS		NS	
4,4'-bis(tripropylene glycol)biphenyl	NS			
Hexane-1,6-diamine	NS		NS	
Dodecane-1,12-diamine	S	T	S	T

Table 1: Solubility and NMR study of six guest molecules. S stands for soluble, NS for not soluble, T for threaded, and S. for solubility.

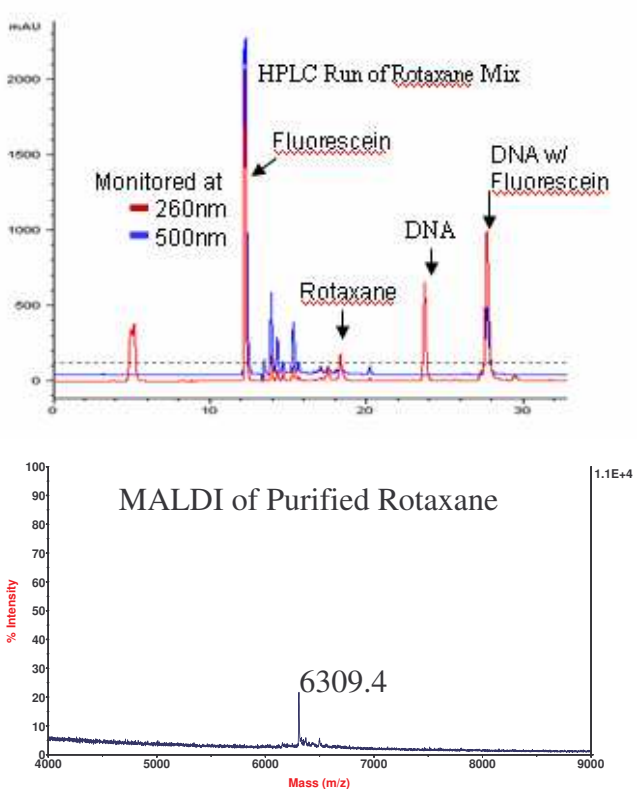

Figure 4: DNA rotaxane was isolated by HPLC and verified with MALDI mass spectrometry. The calculated mass of the rotaxane is 6286.7Da, the spectrum shows the rotaxane with sodium adduct.

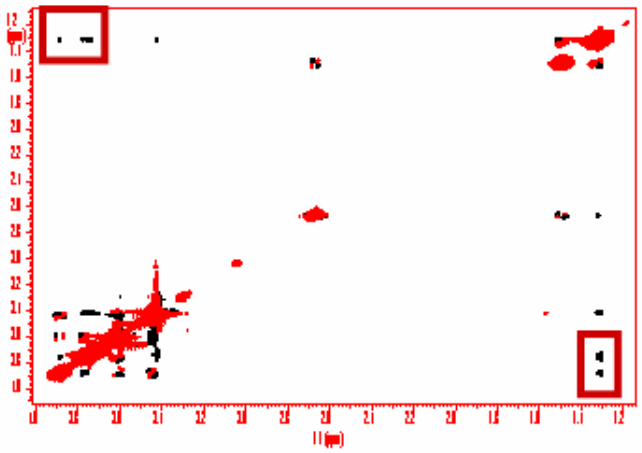

Figure 5: ROSEY of 1,12-diaminododecane threading 2,6-di-O-methyl-β-cyclodextrin.

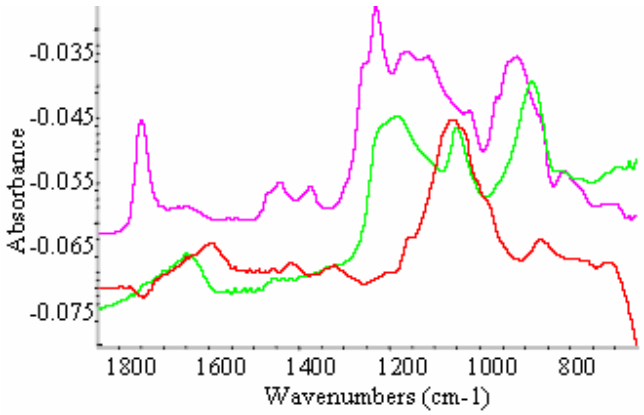

Figure 6: IR of cyclodextrin (green), rinsed adamantane surface (magenta), and stoppered cyclodextrin-adamantane rotaxane.

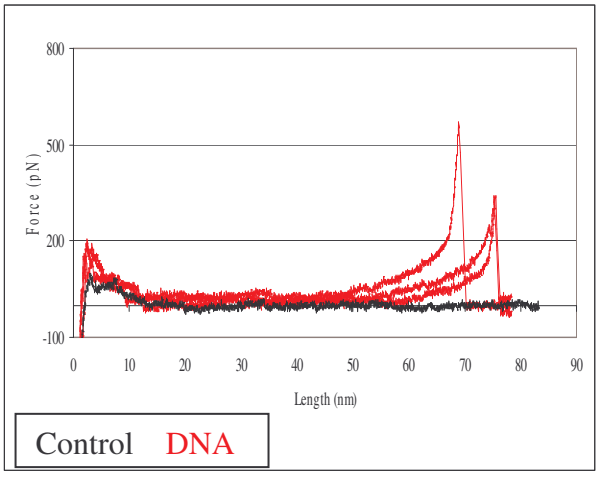

Figure 7: Force curves from stoppered DNA pulling experiment.

4 CONCLUSION

We have successfully completed two of the major pieces needed for our system. The problem of nonspecific adhesion of single-stranded DNA with both hydrophobic and hydrophilic surfaces was overcome by using a poly (ethylene glycol) passivation layer [8]. Two methods to create a DNA-rotaxane were also accomplished.

In order to increase the resolution of our system functional moieties will be attached to the cyclodextrin so it interacts specifically and nonspecifically with the DNA bases. We are also improving the measurement conditions, such as cooling the environment to decrease random thermal fluctuations of the bases. Work is also being done to model the complex behavior of a cyclodextrin sliding over the DNA [9].

ACKNOWLEDGMENTS

The authors would like to thank Ron Nieman and Douglas Klewer for their work on the NMR and John C. Lopez and Daniel Brune for the MALDI results. Funding for this project is from NIH HG003061-01.

REFERENCES

[1] Han, W., Lindsay, S. M., Jing, T. *Applied Physics Letters* **1996**, *69*, 4111.
[2] Saenger, W. *Angew. Chem., Int. Ed. Engl.* **1980**, *19*, 344.
[3] Rojas, M., Koniger, R., Stoddart, J. F., Kaifer, A. E. *J. Am. Chem. Soc.* **1995**, *117*, 336.
[4] Bagheri, M., Didehban, K., Rezvani, Z., Entezami, A. A. *European Polymer Journal* **2004**, *40*, 865.
[5] Cordova, E., Bissell, R. A., Spencer, N., Ashton, P. R., Stoddart, J. F., Kaifer, A. E. *J. Org. Chem.* **1993**, *58*, 6550.
[6] Nepogodiev, S. A., Stoddart, J. F. *Chem. Rev*. **1998**, *98*, 1959.
[7] Kienberger, F., Pastushenko, V. Ph., Kada, G., Gruber, H. J., Riener, C., Schindler, H., Hinterdorfer, P. *Single Mol. 1* **2000**, *2*, 123.
[8] Ashcroft, B. *et al. Langmuir* submitted.
[9] Qamar, S. *et al.* Computational Modeling in Life Sciences Nanotech 06 Proceeding.