Single-molecule Fluorescence and Force Microscopy Employing Carbon Nanotubes

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ABSTRACT

We are developing generalized high-throughput techniques for the growth and attachment of single-wall nanotubes (SWNTs) as robust and well-characterized tools for AFM imaging, to serve as the foundation of an effort to develop single-molecule sensors on nanotube AFM tips for a hybrid atomic force microscope that also has single-molecule fluorescence imaging capability. We have been most successful in attaching SWNTs to AFM tips by growing individual SWNTs on flat surfaces, and using the silicon probe tip to pick up vertically oriented tubes during imaging of these substrates in tapping mode. Once a nanotube has been picked up and shortened, the probe can be transferred to a sample for high-resolution imaging, biomolecular manipulations or force spectroscopy. SWNT AFM images can be used to determine the equivalent resolution to be typically <4 nm for 3 nm diameter SWNTs (noise floor width - tube diameter). This is a factor of two better than is normally achieved with the best conventional silicon tips.

Keywords: Nanotube AFM imaging, Nanotube AFM tip, nanotubes, nanotube growth, nanotube tip

1 INTRODUCTION

The observation of spectroscopic signals, in response to induced changes in biological macromolecules can be enabled at an unprecedented level of resolution by coupling single-molecule manipulation/sensing using carbon nanotubes with single-molecule fluorescence imaging. Proteins, DNA and other biomolecules can be attached to the nanotubes to give highly specific single-molecule probes for the investigation of intermolecular dynamics, the assembly of hybrid biological and nanoscale materials and the development of nanoscale circuitry.

Recent advances in nanotube fabrication and Atomic Force Microscope (AFM) imaging with nanotube tips have demonstrated the potential of these tools to achieve high-resolution images of single molecules[1]. Perhaps the most exciting aspect of SWNTs as AFM probe tips for probing the dynamics of biomolecules is that they can be chemically functionalized uniquely at their very ends. This can be initiated by an electrical etching process, which is also used to shorten the attached SWNTs to achieve lengths suitable for high-resolution imaging. In addition, proof-of-principle demonstrations of nanotube functionalization and attachment of single-molecule probes have been successfully made[2]. Despite the great promise of these tools, there has been little success throughout the community in employing them to conduct broad measurements of real-time *dynamics* that occur within or between individual biological macromolecules. That is primarily because of the difficulty in reproducibly and easily assembling large quantities of single-walled nanotube AFM tips.

The level of resolution possible for both single molecule imaging and force transduction in AFM is ultimately limited by the structure of the tip. Commercially available silicon probe tips have radii of curvature of 5-15 nm. Additionally, cantilevers are typically mounted at some angle relative to the imaging surface (typically ~12°) which causes the face angles of the tips to vary from 0° to 40° relative to normal of the surface being imaged. These tips have not been able to image small detailed structures such as isolated proteins with high resolution[1]. The only exception to this is tips with hyperfine features, which occur on rare occasions[3]. As just noted, variations in tip-to-tip properties are often substantial, which leads to uncertainty in interpreting image or force data due to poorly characterized tip-sample interactions. The finest of these tips are very delicate, leading to substantial variation in tip shape and size even between successive images! For example, in quantifying intermolecular forces and structural dynamics at the single molecule level, there is more often than not uncertainty in the number of molecules interacting with the probe tip, uncertainty in the tip to sample distance and uncertainty in the curvature of the tip. These uncertainties compound to render differentiating specific from nonspecific interactions between the probe and biological samples difficult[4]. The imaging conditions necessary to obtain the highest resolution possible frequently results in damage of soft biological samples and tip wear, which further complicates interpretation of AFM data.

2 NANOTUBE AFM TIPS

Carbon nanotubes (NTs) are in many respects, ideal high-resolution probe tips for AFM[1]. This is particularly true for imaging small objects such as DNA or quantum dots on smooth substrates. Single wall nanotubes (SWNTs) are single atom thick hollow cylinders that are microns in length with typical diameters between 1.6 and 3 nm:

comparable to molecular scale dimensions. Ropes of SWNTs have typical diameters between 5 and 8 nm. Carbon nanotubes are chemically and mechanically robust. They are the stiffest material known, with Young's moduli of about 1.5 TPa, which limits the noise due to thermal vibrations from degrading the ultimate obtainable resolution. Unlike other materials, carbon nanotubes buckle elastically under large loads, limiting damage to both the tips and the sample. Because NTs have well-defined molecular structures, interpreting AFM data becomes much easier since the tip-sample interaction is well characterized and reproducible. Additionally, approximately one-third of pure carbon nanotubes are electrically conductive which is useful for some experiments.

2.1. Nanotube Substrates

Smalley's group reported the first example of the use of carbon nanotubes as AFM tips in 1996[5]. They manually attached MWNTs and nanotube ropes to the apex of silicon pyramidal tips using tape adhesive and a micromanipulator in an optical microscope. The drawbacks to this method are that the mounting process is slow and painstaking, and only large nanotube structures like MWNTs, which can be imaged by the optical microscope, can be employed. These MWNT tips did not improve the resolution much beyond standard silicon tips when imaging isolated amyloid fibrils[6]. Lieber's group later showed that individual single wall carbon nanotubes could be directly grown by CVD on the silicon tips themselves by first pre-coating the tip with a metal catalyst[7-9].

We have concentrated on a pickup technique for attaching nanotubes to silicon AFM tips, which was also developed by Lieber's group[10]. Growth of nanotubes is carried out by CVD on square 500 μm thick Si/SiO_2 wafers with dimensions between 4 mm and 8 mm. Four different methods have been used to successfully coat the silicon substrate with iron catalyst for growing nanotubes suitable for pickup: spin coating with solution of $Fe(NO_3)_3 \cdot 9H_2O$ in isopropyl alcohol[10], Electron Beam evaporation of iron[11], thermal evaporation of iron, and Ferritin[12].

The growth procedure generates primarily SWNTs on the substrate with diameters ranging from 1.6 to 3.0 nm, and lengths between 100 nm and 5 μm, as imaged with SEM and TEM. Some nanotube ropes have also been observed. No multi-walled nanotubes have been observed on these substrates. Most of the tubes are oriented horizontally with respect to the substrate surface, and can be imaged with standard AFM. The results from a typical growth are depicted in Figure 1. Variations in growth density primarily appear to be due to variations in temperature. Note that the ~900 ˚C temperature rise in gas temperature occurs in only ~25 cm. Small changes in position can make a significant change in the final gas and substrate temperature.

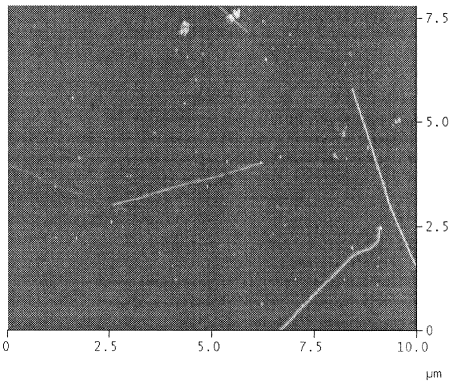

Figure 1: SWNTs grown by CH_4/H_2 CVD on a pick-up (Si/SiO_2) substrate.

2.2 Pickup Attachment of Nanotubes

A small percentage of tubes are oriented vertically, and can be picked up by scanning the AFM cantilever across the surface. Typically, 1 to 4 tubes can be picked up from a 10 μm x 10 μm square region. As seen in figure 2, the tube binds to the side of the pyramidal AFM tip through Van der Waals attractive forces, and usually remains attached firmly enough that it can be repeatedly pressed into or scanned across the substrate surface. The pick-up of a nanotube is readily observed by monitoring the Z height while looking for a significant step change in the average position. In almost all cases, more than 100 nm of nanotube protrudes from the end of the AFM tip, making high resolution imaging impossible due to thermal fluctuations and bending without first shortening the tube to lengths between 25 and 100 nm for a 2 nm diameter SWNT.

Once a SWNT has been picked up and shortened, the probe can be transferred to a sample for high-resolution imaging, biomolecular manipulations or force spectroscopy.

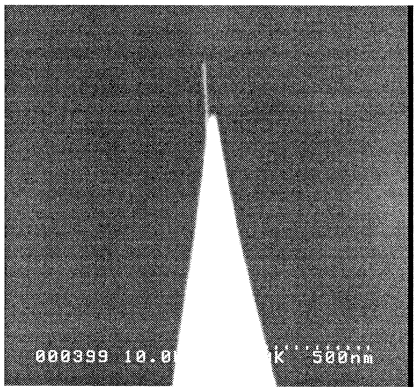

Figure 2: Scanning electron micrograph of an individual, unshortened carbon nanotube mounted on silicon AFM probe. The nanotube was picked up from a flat substrate supporting SWNTs grown by metal catalyzed chemical vapor deposition.

The same SWNT substrate can be used thousands of times to prepare SWNT AFM tips.

2.3 Shortening AFM Nanotube Tips

The length of the nanotube protruding from the end of the AFM tip can be measured indirectly by observing the oscillation amplitude and deflection of the cantilever as it is brought into contact with a hard surface. Consider first the profile of a bare tip in contact with a substrate. As the approaching tip begins to interact with the surface, the oscillation amplitude decreases quickly, reaching zero when the tip is in full contact. At this point of contact, the cantilever deflection signal begins to rise linearly as the tip is pressed further into the surface. In contrast, the oscillation of a tip with a protruding nanotube rapidly damps as soon as the nanotube makes contact with the surface. As the tip is brought closer to the surface, the SWNT buckles elastically at the resultant higher loads and does not significantly deflect the cantilever. Only when the rigid Silicon tip apex itself makes direct contact with the substrate is the deflection detected. The distance between the point at which the oscillation amplitude decreases to zero and the point at which the deflection of the cantilever is detected indicates the protrusion length of the nanotube. In this region, the tube is elastically buckling, which is reversible: when the probe is retracted, the buckled tube can reform to its original shape.

Very short tips (<100nm) can often be push shortened. This approach was developed by Lieber[10]. By taking successive Z-sweep measurements, and incrementing the sweep start point, the tube can be pushed up along the tip. Tubes longer than 100 nm tend to buckle during this process after which they cannot be shortened by further pushing. This is probably the highest reliability method for shortening but requires a picked-up tube of very specific length. This technique is certainly superior when further shortening already short nanotubes in very small increments.

A more general method for shortening utilizes electrical pulses. The procedure we used to shorten nanotube tips in air consists of applying +5 to +30 volt pulses of 20 to 100 μs duration between the AFM tip and a grounded, conductive silver substrate. The pulses are applied when the tip is in contact with a grounded conductive substrate either during tapping mode imaging or while taking a force curve measurement. We used both doped silicon and gold plated silicon substrates with similar results. Presumably the nanotube is shortened by ablation resulting from the very high electric field generated at the nanotube end. For a given nanotube tip, larger voltage pulses shorten the tube in larger increments, as do pulses of longer duration. The voltage necessary to carry out shortening can vary significantly between individual tubes. We believe this variance is due to the widely varying conductivities associated with nanotubes of slightly different molecular structure, for example, between semiconducting and metallic nanotubes and environmental conditions. Nanotubes can be shortened precisely with steps as small as 2 nm per pulse. We find pulse shortening to be superior to push shortening in terms of being able to shorten a nanotube significantly (e.g. by several hundred nm in 20 nm steps).

2.3 AFM Imaging With Nanotubes

Image resolution with nanotubes is dependent on several factors besides the tube diameter. Calculations on the importance of thermal noise and bending of the nanotube (note that the tip surface the tube is mounted on is typically at some angle less than normal to the surface being imaged: causing the tube to bend when pressed against a surface) indicate a need to keep the length of a 2 nm diameter nanotube to ~35 nm to limit errors of ~0.5 nm from each of these error sources[13]. Imaging with longer nanotube lengths is possible but at the cost of reduced resolution. This length constraint significantly restricts the objects that can be effectively imaged to heights less than the nanotube length. In addition a buckled tube will typically result in image artifacts being added. Also the relatively large silicon tip surface will occasionally interact with surface features or films to produce image artifacts. Despite these concerns we have found AFM image qualities to be consistently and significantly better with nanotube tips than with the best silicon AFM tips.

The SWNT AFM image seen in Figure 3 is exceptionally good. The measure width of the noisefloor is only 0.5 nm larger than the measured nanotube diameter. Nanotube tip AFM images more typically demonstrate resolutions between 1 and 4 nm. AFM resolution in the X-Y plane is proportional to the tip radius. Hence the typical resolution demonstrated by nanotubes is approximately 2 to 5 times better than can be achieved with a conventional silicon tip.

3 SINGLE-MOLECULE FLUORESCENCE IMAGING

Coupling single-molecule manipulation/sensing using SWNT AFM with single-molecule fluorescence opens up many possibilities for observing spectroscopic signals in response to mechanically induced changes in biological macromolecules. The two techniques are complementary in many respects. Both are sensitive to conformation changes and movements on the angstrom length scale. However, single-molecule fluorescence can probe chemical kinetics on time scales that may be inaccessible to AFM, from the sub-nanosecond, via single-molecule fluorescence lifetimes, to milliseconds, from changes in orientation or distance using single-pair fluorescence resonant energy transfer or polarization anisotropy measurements. Alternatively, a highly localized mechanical perturbation at a specific binding site on a protein, utilizing a AFM with a functionalized SWNT probe, can generate profound

changes in the mechanism of an active site located further away on the macromolecule, characterized with single-molecule fluorescence measurements of the kinetics. Specific examples of ways that single-molecule manipulation techniques can be used simultaneously with single-molecule fluorescence to sensitively probe the structural dynamics of complex biological systems include controlling ligand/receptor binding or substrate/enzyme interactions, the manipulation of one component of a biomolecular motor relative to another, and mechanically tuning the interactions between individual proteins involved in a cellular signaling pathway. There are examples of hybrid single-molecule fluorescence, single-molecule nano-manipulation studies reported in the literature[14], although to the best of our knowledge, none have been performed using SWNT tips as the mechanical manipulation and sensing tools.

Single-molecule manipulation methods, involving the use of glass microneedles, optical tweezers and atomic force microscopy (AFM) enable the detection and, in some cases, control, over the mechanical motions, forces and strains in a biological system as a reaction proceeds[15]. The combination of single-molecule imaging *and* single-molecule manipulation techniques represents a powerful and versatile approach for probing biomolecular dynamics that is more than the sum of its parts. Simultaneous measurements of spectroscopic and mechanical signals from single biomolecules, and the changes induced in chemical kinetics from externally controlled molecular movements have become possible[14].

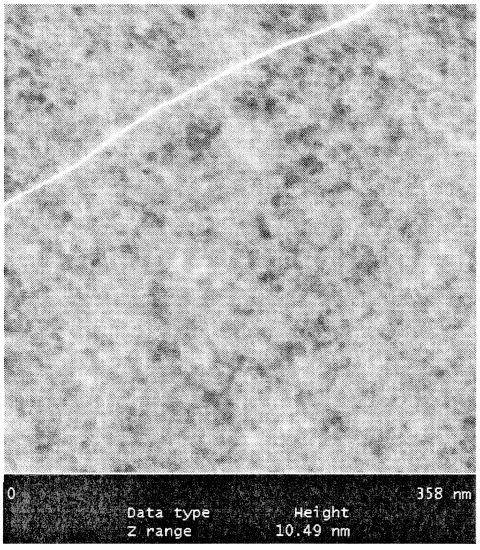

Figure 3: AFM images of a SWNT on an oxidized silicon substrate taken using a shortened pickup nanotube tips. The height of the SWNT is measured as 1.6 nm and the width is 2.1 nm at the noise floor. This is an exceptionally good image. The resolution, as defined by the difference between the noise floor width and the height, is frequently found to be 1.5 to 3 nm.

4 SUMMARY AND CONCLUSIONS

AFM imaging with nanotube tips suitable for imaging dry samples with very high-resolution has been developed. These tips are suitable for imaging small objects typically <30 nm in height on smooth substrates such as silicon, mica and glass. Resolution with these tips is typically < 4nm and rarely as good as 0.5 nm when imaging nanotubes <3 nm in diameter. Nanotubes were attached to the silicon AFM tips by the pick-up technique and then shortened by push, pulse or both in combination. The processes used are sufficiently high productivity to enable development of single molecule probes and sensors using functionalized nanotube tips. After doing so we will be able to couple single-molecule fluorescence detection with SWNT AFM imaging.

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